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Degree: Master of Science

Year this Degree Granted: 2001

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University of Alberta

A positive Kähler indicatrix in Finsler geometry

by

Julie Jeannine Klassic

"Faith is to believe what you do not yet see;
the reward for this faith is to see what you believe."

A Thesis submitted to the Faculty of Graduate Studies and Research in
Partial Fulfillment of the requirements for the degree of Master of Science

Mathematics and Statistics

Department of Mathematical Sciences

Edmonton, Alberta

Fall, 2001

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **A putative RNA helicase in *Streptomyces coelicolor*** submitted by **Julie Jeannine Stoehr** in partial fulfillment of the requirements for the degree of **Master of Science** in **Microbiology and Biotechnology**.

Abstract

A partial open reading frame (ORF) has been identified in *Streptomyces coelicolor* that is transcribed divergently from *bldG*, and the deduced amino acid sequence indicated that this ORF, designated SCH5.13, encodes a putative RNA helicase. Transcriptional analysis of the SCH5.13 ORF revealed a temporal expression pattern with increased transcript levels both during vegetative growth and at the onset of sporulation in *S. coelicolor*, indicating that this putative RNA helicase could play a dual role in this organism. Localization of the SCH5.13 transcription start point has indicated that the promoter for SCH5.13 overlaps the previously identified *bldG* promoters, thus suggesting that expression of the putative RNA helicase and *bldG* could involve a coordinate control mechanism. Although attempts to isolate an SCH5.13 null mutant in a wild type *S. coelicolor* background failed, this putative helicase gene could be disrupted in a *bldG* mutant strain, prompting the suggestion that at least one role of this enzyme could be to suppress sporulation during vegetative growth.

Acknowledgements

First, I must start by thanking, not only my supervisor, Dr. Brenda Leskiw, but also her husband Dan. Together they have shown me a love of science, the joy of teaching and the ability to "have it all" in life. They are terrific role models and I thank them both not only for the academic support I have received but also the countless moments of personal enjoyment.

I would also like to extend my appreciation to Dr. Owttrim for his expertise and suggestions throughout the course of my project. Dr. Laura Frost has been a terrific mentor not only as a member of my supervisory committee but also as my teaching supervisor for three years – thank you for your faith in my ability even in the beginning when things were a little rough! In this vane I would be remiss if I did not thank Tom Hantos who has been nothing but supportive, technically and personally (if not a little mischievous) for the duration of my project.

I would never have made it to the end if not for the members, past and present, of the Leskiw Lab. I especially would like to thank Dawn Bignell and Marie Elliot who have not only been excellent lab-mates, but who I feel very honoured to consider my friends. I owe Kent Gislason a huge thank you for his help in the last legs of my project and I wish him luck in discovering new and exciting things as he takes on graduate school (and my project). Thank you to all members of my family – immediate and extended – your support and excitement for my accomplishments is contagious!

Finally, I could not have done anything without the love and support of Kevin Arsenault who is one of the most patient and understanding individuals I have ever had the good fortune of knowing. I don't know what I did to deserve you but I am grateful and I love you.

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List of Abbreviations

α	Alpha
β	Beta
γ	Gamma
λ	Lambda bacteriophage
ϕ	Phi
σ	Sigma factor
A	Adenine or Alanine
Ap^R	Apramycin resistance gene
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine (DNA) or cysteine
C-	Carboxyl
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
cpm	Counts per minute
D	Aspartate
ddNTP	Dideoxy nucleoside triphosphate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxy nucleoside triphosphate
DTT	Dithiothreitol
E	Glutamate
EDTA	Ethylenediamenetetraacetic acid
eIF-4A	Eukaryotic initation factor – 4A
G	Guanine (DNA) or Glycine
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
H	Histidine
HCV	Hepatitis C virus
HEPES	Hydroxyethylpiperazinethansulfonic acid
I	Isoleucine
IPTG	Isopropyl β-D-thiogalactopyranoside

K	Lysine
kb	kilobase
L	Leucine
LB	Luria Bertani medium
MOPS	3-(N-morphino)propane sulfonic acid
mRNA	Messenger RNA
MW	Molecular weight
N-	Amino
nt	Nucleotide
NTG	Nitrosoguanidine
NTPase	Nucleoside triphosphatase
NPH-II	Nucleosode triphosphate phosphohydrolase
NTP	Nucleoside triphosphate
ORF	Open reading frame
P	Proline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsulfonyl fluoride
ppGpp	Guanosine tetraphosphate
pre-mRNA	
Q	Glutamine
R	Arginine
R2YE	Sucrose yeast-extract medium
RBS	Ribosome binding site
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RNPase	Ribonucleoprotein displacement activity
rpm	Revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Serine
SDS	Sodium dodecyl sulfate
SF2	Superfamily 2
SSC	Standard saline citrate
ssRNA	Single stranded ribonucleic acid

T	Thymine (DNA) or Threonine
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
TEMED	Tetramethyl elthylene diamine
Tm	Melting temperature
tRNA	Transfer RNA
TSB	Trypticase soy broth
tsr	Thiostrepton resistance gene
U	Unit (measure of enzyme activity)
UTP	Uracil triphosphate
UTR	Untranslated region
UV	Ultraviolet
w/v	Weight per volume
x-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
YEME	Yeast extract malt extract medium

Chapter 1:

Introduction

1. INTRODUCTION

The Gram positive, filamentous soil bacteria belonging to the genus *Streptomyces* are well known for their complex cycle of morphological differentiation. This life cycle, elaborate by prokaryotic standards, involves the formation of structures that are well adapted for survival in the harsh environment in which these organisms live (Fig. 1.1). The morphological cycle begins with the germination of a single spore that develops into a vegetative substrate mycelium that can extend into a solid growth medium. The substrate mycelium imparts a shiny appearance to the colony and is ideal for scavenging limited nutrients as it can penetrate deep into the surrounding environment and secretes numerous hydrolytic enzymes. As this structure possesses few cross walls, the vegetative hyphae also contain numerous copies of the streptomycete genome. This multi-nucleate mycelium will continue to grow until an as yet undetermined signal, probably nutrient limitation, causes a cessation in vegetative growth. A halt in substrate mycelial growth triggers the onset of a cascade of regulatory signals leading to the erection of aerial hyphae, imparting a fuzzy white appearance to the colony surface. Aerial growth requires the hydrolysis of glycogen storage granules that accumulate at the substrate mycelium – air interface, allowing sufficient turgor pressure needed to allow the hyphae to break the surface tension at the colony surface (Plaskitt and Chater, 1995). The remaining stages of the *Streptomyces* life cycle involve hydrolyzing a second batch of glycogen granules which collect at hyphal tips to allow coiling of the multi-genomic aerial hyphae and formation of unigenomic pre-spore compartments (Plaskitt and

Figure 1.1: *Streptomyces coelicolor* life cycle. Genes indicated control specific stages of development.

Germination

Substrate Mycelium

bldA-N

Aerial Hyphae

Single Spore

bldG mutant strain

whiG, whiJ

Spore Formation

whiA, whiB

Grey Mature Spores

whiD, sigF, whiE

whiH, whiI
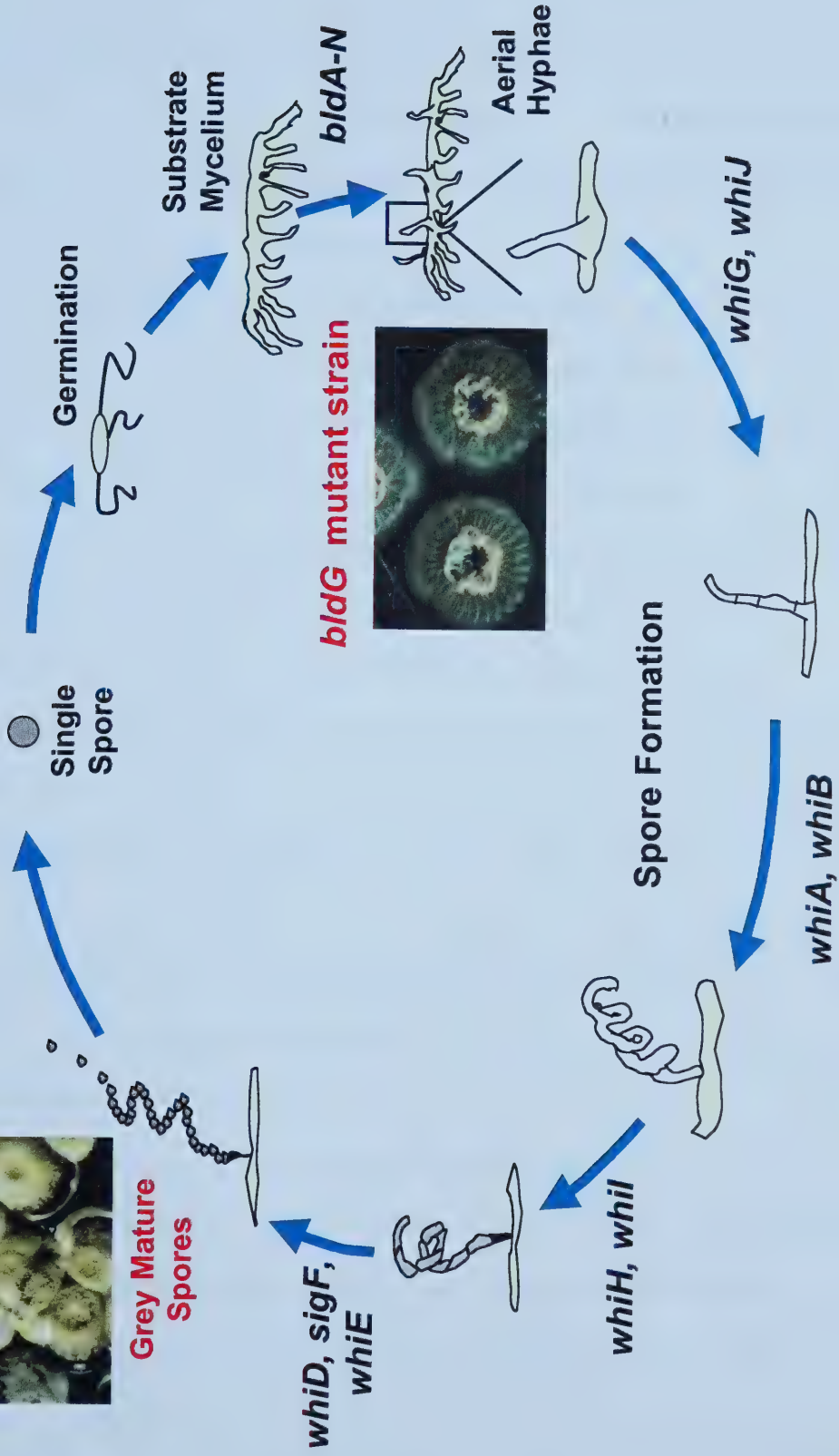
Chater, 1995). At this point, chromosomal DNA condenses, pre-spore cross-walls thicken and finally a grey polyketide pigment coats the heat and desiccation resistant spores. These spores are now able to be released into the environment and remain dormant until such time as conditions allow their germination and the cycle of development can begin anew.

Interestingly, at the time of aerial growth initiation, the substrate mycelium produces numerous secondary metabolites. The secondary metabolites include various antibiotics, anti-fungals, herbicides, immunosuppressants, and anti-tumour compounds that have been exploited by pharmaceutical companies to result in an industry worth nearly $14 billion dollars (U.S.) annually. Because of their clinical and environmental significance, many of the biosynthetic gene clusters responsible for antibiotic production have been characterized. Regulation of the physiological differentiation (antibiotic production) process exists on many levels, including pathway specific activation of synthesis, global antibiotic regulators, and pleiotropic regulators that tie this process to morphological development. It is this connection to morphological differentiation that has more recently been examined and the elaborate regulatory networks responsible for these morphological and physiological changes have been the subject of much research.

The striking transformation between multinucleated aerial hyphae and grey, mature spores is brought about by the activity of the *whi* (for white) genes which were initially isolated and named as a description of their mutant phenotype (Chater *et al.*, 1989). When mutated, *whi* genes cause an arrest in

growth at the aerial mycelium stage resulting in colonies bearing a fluffy, white appearance. Generating a great deal of excitement has been the discovery, through cloning and characterization, that most of the *whi* genes encode regulatory proteins rather than enzymatic or structural proteins. At present, thirteen *whi* genes have been identified (Chater, 1972; Ryding *et al.*, 1999) nine of which, based on the stage of blockage in sporulation, have been placed into a hierarchy: *whiG,whiJ* < *whiA,whiB* < *whiH* < *whiI* < *whiD,sigF,whiE*. As a further division, these genes have also been classified into early and late *whi* genes. Early *whi* genes, which are responsible for the formation of sporulation septa, define six loci: *whiA*, *whiB*, *whiJ*, *whiG*, *whiH* and *whiI* (Chater, 1972), all of which have been cloned, and most of which have been at least partially characterized.

Mutants in *whiG* are blocked at the earliest stage in sporulation, only aerial hyphae that have not undergone any coiling or septation are produced, thus this gene represents the first step or signal in the transition between aerial hyphae and spore formation (Chater *et al.*, 1989). *whiG* encodes a sporulation-specific σ-factor, aptly named σ^{WhiG} (Chater *et al.*, 1989; Tan and Chater, 1993). The presence of a sigma factor is significant because it demonstrates differential promoter selection by an alternate sigma factor as part of the sporulation strategy, a tactic that has been described in other bacteria for coping with various environmental stresses (Kalman *et al.*, 1990). Interestingly, σ^{whiG} shows highest homology to σ^{D} of *Bacillus subtilis*, which belongs to the family of motility sigma factors instead of the sporulation family (Chater *et al.*, 1989). Transcriptional studies of *whiG* have revealed that the *whiG* promoter is active at a relatively

constant level throughout the life cycle, instead of in a growth – phase dependent manner as would be expected for a gene involved in the later stages of differentiation (Kelemen *et al.*, 1996). This has led to the suggestion of a post-transcriptional control mechanism for *whiG* function, possibly in the form of an anti-sigma factor / anti- anti sigma factor regulatory pair. The best characterized examples of this system for regulation of gene transcription have been described in *Salmonella* (σ^{FliA}) (Ohnishi *et al.*, 1990) and *Bacillus subtilis* (σ^{F}) (Duncan and Losick, 1993). Usually the anti-anti sigma factor, the anti sigma factor, and the sigma factor are transcribed as an operon. Unfortunately, neither an anti- anti sigma factor nor an anti sigma factor are encoded in close proximity to the *whiG* gene.

whiB is a small, cytoplasmic protein whose predicted structure suggests a role in transcription regulation of sporulation (Davis and Chater, 1992). This protein contains four cysteine residues that could interact with metal ions or form disulfide bridges, indicating a possible role in sensing the redox state (Davis and Chater, 1992). This feature is an indication that the organism might react to signals resulting from metabolic changes that occur as hyphal growth ceases. In the same hierachial group as *whiB* is the recently characterized *whiA*. This gene has been found to encode a protein with orthologues in all Gram positive bacteria whose genome sequences are currently known (Ainsa *et al.*, 2000). None of the known WhiA orthologues has been assigned a definite function, however it has been suggested that *whiA* and *whiB* function together to facilitate the cessation of aerial growth (Flardh *et al.*, 1999). Both *whiA* and *whiB* are transcribed from

multiple promoters (Ainsa *et al.*, 2000; Soliveri *et al.*, 1992) and strangely, neither is dependent on σ^{whiG} for transcription despite *whiG* mutations being epistatic to *whiA* and *whiB* (Flardh *et al.*, 1999; Ainsa *et al.*, 2000).

Unlike *whiA* and *whiB*, the remaining two characterized early *whi* genes, *whiH* and *whiI* are directly dependent on transcription by the σ^{whiG} form of RNA polymerase (Ainsa *et al.*, 1999; Ryding *et al.*, 1998). The recently characterized *whiI* encodes an atypical response regulator protein, which has demonstrated a weak auto-repression activity (Ainsa *et al.*, 1999). In addition, a *whiI* null mutant shows a de-repression in *whiH* transcription, hinting at a cross-regulatory network between these two proteins. *whiH* encodes a protein belonging to a family of regulatory proteins that act as repressors and whose activity can be relieved in response to carboxylate-containing metabolic intermediates (Ryding *et al.*, 1998; Chater, 1998). It is suggested that WhiH repression of certain promoters can be relieved once the concentration of an as yet unknown carbon metabolite increases such that it can interact with the WhiH protein (Ryding *et al.*, 1998). An interesting observation is that *whiH* mutants are not abnormally long (as in *whiA* and *whiB*), indicating an ability to retain proper control of the decision to stop aerial growth (Flardh *et al.*, 1999). Together with the observation that these mutants also produce small amounts of spore pigment and few sporulation septa, these phenomena have led to the suggestion of developmental checkpoints in the sporulation process controlled by *whiG* and by *whiA* and *whiB* (Flardh *et al.*, 1999).

The latter stages in sporulation, consisting of spore maturation and pigment production, are under the control of the aptly named "late" *whi* genes, *whiD*, *sigF*, and *whiE*. Just as the early sporulation events depend on the expression of σ^{whiG}, the late sporulation genes depend on the expression of *sigF*, the gene encoding a sporulation specific sigma factor (Potuckova *et al.*, 1995). Expression of *sigF* is dependent, although indirectly, on the σ^{whiG} directed RNA polymerase. Because σ^{WhiG} does not directly transcribe *sigF*, it is possible that control of the late sporulation sigma factor could be exerted through an as yet undiscovered sporulation-specific σ factor that is itself under transcriptional control of *whiG* (Kelemen *et al.*, 1996). The *whiD* gene product is part of the growing family of proteins present in many actinomycetes that includes the early *whi* gene *whiB* (Molle *et al.*, 2000). As yet, the proteins have no characterized function, however the presence of the above-mentioned four cysteine residues in all members of this protein family has been suggested to indicate a role in redox sensing. The *whiE* gene cluster encodes a Type II polyketide synthase, therefore it is understandable that mutants in the *whiE* locus produce nearly wild-type spores lacking only the grey, polyketide spore pigment (Kelemen *et al.*, 1998). It has been suggested that the *whiE*-encoded pigment provides a carbon "sink" for the products of glycogen metabolism that is needed to initiate spore formation, however, this hypothesis has not been unequivocally confirmed (Plaskitt and Chater, 1995).

The second group of morphological mutants originally isolated (Merrick, 1976) were classified as the *bld* (for bald) mutants which have a striking

phenotype. Mutations in the genes categorized as *bld* result in complete lack of aerial mycelium and in most cases a loss of antibiotic production. In *S. coelicolor* these mutants are the most noticeable because two of the antibiotics produced normally by this species are pigmented. Actinorhodin is a blue polyketide pigment that diffuses from the colonies into the surrounding medium (Bystrykh *et al.*, 1996) while undecylprodigiosin is a red tripyrrole antibiotic that remains cell-associated (Tsao *et al.*, 1985). The inherent production of these coloured compounds makes *S. coelicolor* and ideal organism in which to study genes encoding regulators of antibiotic production, such as *bld* genes because the absence of antibiotic production is visibly noticeable.

The apparent link between metabolism and development that has been alluded to with *whiH* was first proposed by Pope *et al.* (1996) when it was discovered that *bld* mutants were deficient in carbon utilization and catabolite repression. Most *bld* mutants will develop aerial hyphae and sporulate when grown on mannitol as the sole carbon source (Champness, 1988; Merrick, 1976). This has suggested that there should be two alternative pathways for differentiation in *Streptomyces*, one that is glucose repressible and one that is dependent on *bld* gene function. It has long been known that a nutritional shift-down, resulting in accumulation of ppGpp, causes an increase in actinorhodin production (Hesketh *et al.*, 2001), and mutants in *relA* (ppGpp synthase) are bald under nitrogen limiting conditions (Chakraburtty and Bibb, 1997). That metabolic defects are a factor in differentiation has been further validated through studies by Süsstrunk *et al.* (1998) using mutations in the adenylate cyclase gene, *cya*.

Mutants incapable of synthesizing cAMP are unable to produce aerial mycelium or antibiotics and irreversibly acidify the surrounding medium. Additional evidence that medium acidification leads to developmental growth arrest in *S. coelicolor* has been found in studies of mutations made in two TCA cycle enzymes, aconitase (*acoA*) (Viollier *et al.*, 2001), and citrate synthase (*citA*) (Viollier *et al.*, 2001). These mutations result in accumulation of extracellular organic acids which leads to the inability to erect aerial hyphae. Indeed, the citrate synthase mutant strain does show classical bald characteristics, including fitting into the proposed extracellular *bld* gene cascade described below (Viollier *et al.*, 2001). However, metabolic dysfunction cannot be the only explanation because neither increasing the medium pH, nor supplying exogenous cAMP to classic *bld* mutants restored a wild type phenotype (Süsstrunk *et al.*, 1998).

Extracellular complementation studies by Willey *at al.* (1991) revealed that *bld* mutants could be morphologically and physiologically rescued by wild-type colonies grown nearby. Further investigation into this phenomenon resulted in the identification and isolation of a small, morphogenetic protein, SapB, which diffused through the medium between colonies. SapB is a 2 kDa, hydrophobic peptide that, when applied exogenously to *bld* mutant colonies (Willey *et al.*, 1991; Willey *et al.*, 1993), can restore aerial hyphae formation. However, the restoration of aerial hyphae formation by this method is transient, and does not allow full differentiation into spores, nor antibiotic production, to be rescued. SapB's inability to fully complement the *bld* mutant phenotype was explained once the nature of this protein was elucidated. Tillotson *et al.* (1998) concluded

that SapB is, in fact, a surface active protein that has similar function to fungal hydrophobins, and acts to reduce the colony surface tension and allow otherwise vegetative hyphae to extend upwards.

In addition to being complemented by nearby wild type colonies, it was discovered that pairs of *bld* mutants themselves could unidirectionally complement one another, such that a "donor" mutant strain could restore differentiation capabilities of a "recipient" mutant strain, but not vice versa. The pattern of rescue that was discovered led Willey *et al.* (1993), to propose a *bld* gene hierarchy as follows: *bldJ* < *bldK* < *bldA,bldH* < *bldG* < *bldC* < *bldD,bldM* in which the *bld* mutants to the left are able to rescue all mutants to the right. It was proposed that this hierarchy culminated in the production of SapB, which diffused through the medium, thus allowing complementation. However, the inability of SapB alone to fully reinstate morphological and physiological differentiation, in contrast to the complete rescue by the *bld* mutant and wild type strains, has led researchers to suggest that a cascade of extracellular signals exists (Willey *et al.*, 1993). Not included in the above hierarchy are a number of *bld* genes (*bldB*, *bldI*, *bldN*) that do not conform to a single complementation group. The inability to place these genes into a defined complementation group is an indicator that the proposed signaling hierarchy is more complex than originally anticipated.

In the simplest of scenarios, the *bld* genes in the hierarchy would encode either the signals of the hierarchy or components for signal sensing or uptake, whose sequential expression would result in aerial mycelium formation and antibiotic production (Fig. 1.2). In fact, there is some evidence to support this

Figure 1.2: A model for a regulatory signal cascade in *S. coelicolor*. The *bldJ* – dependent signal (1) is produced and accumulates extracellularly at which point it is imported by the *bldK* – encoded oligopeptide permease. Uptake of signal 1 triggers synthesis and release of signal 2, and so on, culminating in the *bldD* – dependent production of SapB and thus aerial hyphae.

(Modified from Kelemen and Buttner, 1998)
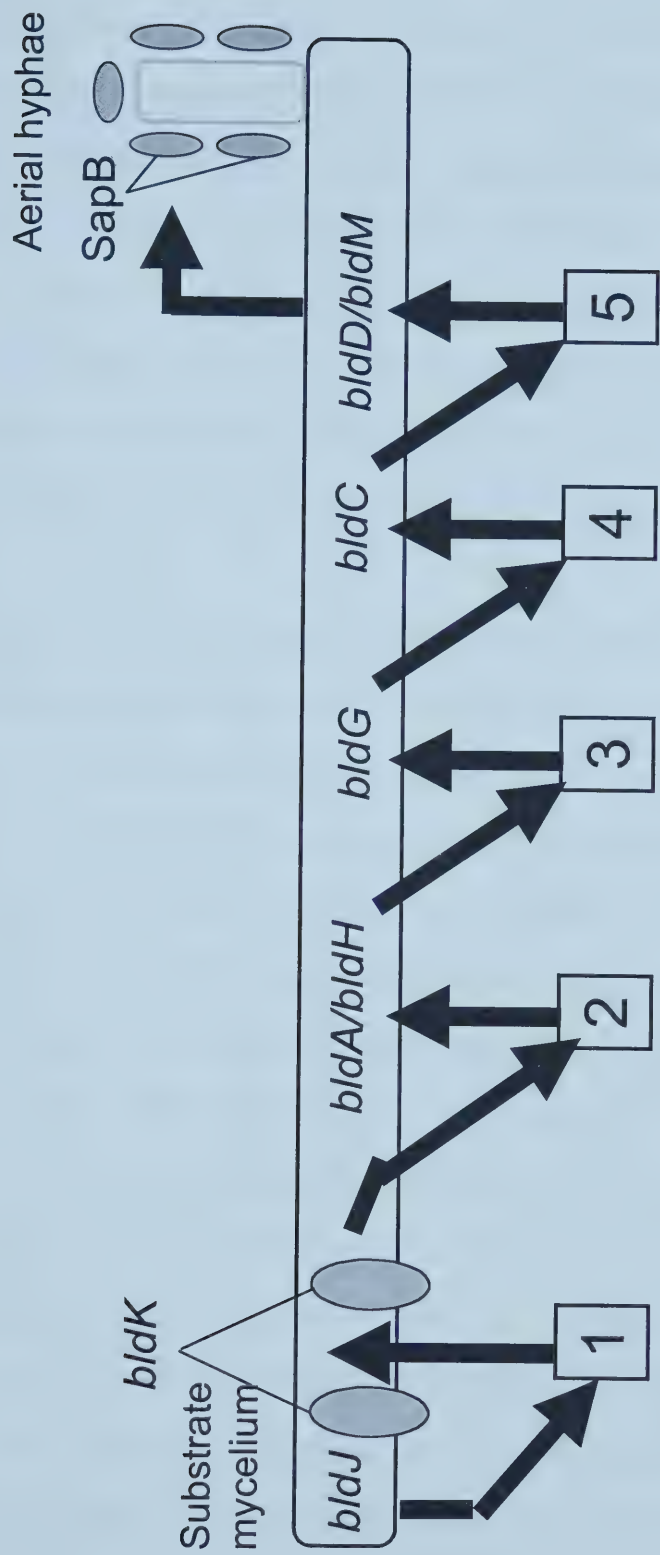
theory as *bldK* has been demonstrated to encode a locus of five components of an ATP-dependent oligopeptide permease (Nodwell *et al.*, 1996), which is presumed to import the *bldJ* (formerly *bld261*) dependent signal (Nodwell and Losick, 1998). This glycine and serine rich oligopeptide was isolated by Nodwell (1998) and is a solid piece of evidence supporting the idea of a cascade of *bld* gene dependent signals. Upon examination of the remaining *bld* gene products however, it has become quite clear that there must be numerous indirect regulators of differentiation that exert their effects within a more complex network of signals.

The most characterized *bld* gene to date is *bldA*, which appears to be a central pleiotropic regulator that encodes a leucyl-tRNA, recognizing the rare UUA codon in *Streptomyces* mRNA, (Lawlor *et al.*, 1987). In an organism with a G+C% of greater than 70%, TTA codons are rare and are generally present only in those genes required for differentiation processes (Leskiw *et al.*, 1991b). Although transcription of *bldA* occurs constitutively, accumulation of the 5' processed form of the tRNA does not increase until later in the growth cycle (Leskiw *et al.*, 1993). This results in inefficient translation of UUA-containing mRNAs until differentiation occurs, thus imposing a translational regulation element in those genes required for translation. Two genes responsible for suppressing untimely morphological differentiation in *S. griseus*, *amfR* (Ueda *et al.*, 1993) and *nrsA* (formerly *orf1590*) (Kwak and Kendrick, 1996; McCue *et al.*, 1996) also contain TTA codons. However, in order to suppress premature sporulation, both of these transcripts must be translated during vegetative

growth. This does occur, albeit inefficiently, yet the necessity of imposing this translational control on genes required early on is unclear. Homologues of both genes are known in *S. coelicolor* although *ramR*, the homologue of *amfR*, does not contain a TTA codon (Ma and Kendall, 1994). BldA also exerts its control over antibiotic production through translational regulation of pathway specific activators. The activator of actinorhodin production, *actII*ORF4, contains a TTA codon (Bystrykh *et al.*, 1996; Passantino *et al.*, 1991) as does the *redZ* gene, which activates transcription of *redD*, the pathway specific regulator of undecylprodigiosin synthesis (Guthrie *et al.*, 1998; White and Bibb, 1997). Thus, the temporal control of *bldA* –encoded tRNA processing and the infrequent use of TTA codons impose a translational control mechanism on genes required for morphological and physiological differentiation in numerous streptomycetes.

The product of the *bldB* gene encodes a small putative DNA binding protein that apparently negatively regulates its own expression (Harasym *et al.*, 1990; Pope *et al.*, 1998). The *bldB* mutant is one of a small number of the *bld* mutants that is not rescued by growth on poor carbon sources such as mannitol (Pope *et al.*, 1996). As yet, no targets have been identified for BldB.

Similar to BldB, the *bldD* gene encodes a DNA binding protein that exerts transcriptional control over a number of developmentally significant genes including expression from its own promoter (Elliot *et al.*, 1998; Elliot and Leskiw, 1999). To date, three sigma factors involved in sporulation (σ^{WhiG}), aerial hyphae formation (σ^{BldN}) and the stress response (σ^{H}) (Kelemen *et al.*, 2001) are known to be directly repressed at the level of transcription by BldD (Elliot *et al.*, 2001;

Kelemen *et al.*, 2001). The interaction with the stress response sigma factor gene *sigH* maintains the idea that *Streptomyces* species sense environmental changes and thus possess a global connection between stress and development. To further validate the hypothesis, examination of stress regulon induction by Vohradsky *et al.* (2000) revealed that many stress response proteins, such as those involved in heat and salt shock, are under developmental control. More recently, oxidative stress has also been implicated in development through the discovery and characterization of developmentally regulated catalases (Cho *et al.*, 2000). The connection of stress response with the *bld* gene cascade (through regulation by *bldD*) however, is an exciting step in establishing how these complex organisms utilize environmental sensors to initiate differentiation.

BldD directly and indirectly influences transcription of *bldN* and *bldM* respectively (Bibb *et al.*, 2000; Elliot *et al.*, 2001; Molle and Buttner, 2000). Both *bldM* and *bldN* were originally classified as *whi* genes due to the mutant phenotypes observed in the original screen for NTG-induced mutants (Ryding *et al.*, 1999). However, upon construction of null mutants, *bld* phenotypes resulted, thus *whiK* was renamed *bldM* (Molle and Buttner, 2000) and *whiN* was renamed *bldN* (Bibb *et al.*, 2000). *bldN* is a recently characterized gene whose product is an extracytoplasmic function (ECF) sigma factor responsible solely for the production of aerial hyphae (Bibb *et al.*, 2000). Thus, mutants in *bldN* do not have a "classical" bald phenotype as they do produce antibiotics. The RNA polymerase directed by σ^{BldN} is responsible for transcription from the p1 promoter of *bldM*, which encodes a member of the FixJ subfamily of response regulators

(Molle and Buttner, 2000). *bldM*, understandably, affects only aerial hyphae formation as well, and has been added to the *bld* gene cascade as a member of the *bldD* complementation group (Molle and Buttner, 2000). ·

The gene product of *bldG* is a putative anti-anti sigma factor that is transcribed both as a monocistronic transcript and as part of a polycistronic transcript with a downstream gene designated *orf3* (Bignell *et al.*, 2000). The *orf3* gene encodes a putative anti sigma factor. Together, BldG and ORF3 closely resemble components of the *rsb* operon necessary for stress response in *B. subtilis* (Benson and Haldenwang, 1993) and the *spoIIA* operon that directs sporulation in *B. subtilis* (Duncan and Losick, 1993). However, where the *Bacillus* systems consist of an operon of genes encoding the anti-anti sigma factor, the anti sigma factor and the regulated sigma factor, this novel system in *S. coelicolor* lacks a gene encoding a sigma factor (Bignell *et al.*, 2000). Instead, transcribed divergently from *bldG* is an open reading frame whose deduced amino acid sequence closely resembles several known and putative RNA helicases.

RNA helicases are enzymes that utilize ATP (or other nucleoside triphosphates) to unwind, or destabilize RNA duplexes that are often the result of secondary structure formation in otherwise single stranded RNA. These proteins are an intricate part of numerous cellular processes including, translation initiation, mRNA degradation, ribosome biogenesis, pre-mRNA splicing, and localization of signals required for development and differentiation. The nucleic acid destabilizing enzymes encompass a large group of proteins found in

prokaryotes, eukaryotes, viruses and archae that are involved in replication, repair and recombination of both DNA and RNA. Based on amino acid sequence similarity, helicases have been classified into three large superfamilies and two smaller families (Gorbalenya and Koonin, 1993). It is the superfamily 2 (SF2) to which the RNA helicases belong (Gorbalenya *et al.*, 1989).

Because of the conserved Asp-Glu-Ala-Asp (D-E-A-D) motif found in RNA helicases, these enzymes are often referred to as "DEAD-box" proteins (Linder *et al.*, 1989), however in actuality, variations on this motif do occur. These modifications include the DEAH and the DExH motifs, which ultimately results in the RNA helicase enzymes being subdivided into three subfamilies (DEAD, DEAH, and DExH) based solely on this motif (Pause and Sonenberg, 1992; Schmid and Linder, 1992). Members of the helicase superfamily 2 contain up to eight conserved amino acid motifs with relatively conserved spacing found between them (Fig.1.3). All RNA helicases posses the well-characterized GxGKS/T, DEAD, SAT, and H/QxxGRxxR motifs, and many contain additional conserved sequences such as PTRELA, GG and TPGR (Pause and Sonenberg, 1992). Because they are so highly conserved among such a wide variety of proteins, the helicase motifs have been subjected to mutational analysis in order to better elucidate their functions in enzyme activity.

The Walker motif "A" (GxGKS/T) is conserved not only in RNA helicases but is of vast importance for all NTP-utilizing enzymes (Walker *et al.*, 1982). It has been concluded that this motif is required for binding ATP and that the lysine

Figure 1.3: Schematic representation of DEAD, DEAH and DExH subfamilies of RNA helicases. Boxed regions indicate conserved amino acid motifs given in single letter notation. Numbers indicate spacing between motifs as adapted from Pause and Sonenberg (1992).

DEAD Family:

NH$_2$ — [AXXXXGKT] — [PTRELA] — [GG] — [TPGR] — [DEAD] — [SAT] — [ARGXD] — [HRIGRXXR] — COOH

21-299   24-42   22-28   19-27   19-22   27-51   115-137   20   24-236

DEAH Family:

NH$_2$ — [GXXXXGKT] — [PRRVAA] — [XX] — [TDGX] — [DEAH] — [SAT] — [XXGXX] — [QRIGRXGR] — COOH

245-505   22-24   29   7-8   19   28   136-149   25   315-373

DExH Family:

NH$_2$ — [XXXXXGKT] — [PTRXXX] — [DEXH] — [TAT] — [XXGXX] — [QRXGRXGR] — COOH

83-1904   19-27   55-60   24-30   70-80   38-44   155-1799

residue is particularly important for the interaction probably due to contact made with the phosphates of ATP (Pause and Sonenberg, 1992; Rozen *et al.*, 1989). The DEAD motif (or one of its modified versions) is a variation of the Walker "B" motif of NTPases (Walker *et al.*, 1982). This motif has been deemed more important for ATP hydrolysis than for binding and may also be involved in coupling hydrolysis of ATP to unwinding of RNA duplexes. It is interesting that a substitution of the second aspartate with a histidine (i.e. DEA*H*) results in a decrease in helicase activity despite a noticeable increase in ATPase activity (Pause and Sonenberg, 1992). There is the possibility that members of the DEAH and DExH subfamilies compensate for this outcome through a variation in the HRIGR motif discussed below.

Where the Walker motifs A and B are ubiquitous in NTP binding and hydrolyzing proteins, the SAT motif is a unique sequence found only in members of the SF2 (Gorbalenya *et al.*, 1989). Originally, mutational analysis of this motif was done on the prototypal RNA helicase, eukaryotic initiation factor 4A (eIF-4A) and alterations in either the serine or threonine residues caused a loss of RNA unwinding activity while ATPase and ATP binding remained comparable to the wild type (Pause and Sonenberg, 1992). Later, the same effect was demonstrated in NPH-II, an RNA helicase of the DExH subfamily from vaccinia virus (Gross and Shuman, 1998), and more recently mutations in the SAT motif of the DEAH protein Prp22p of *Saccharomyces cerevisiae* confirmed that the two hydroxyamino acids are pivotal in coupling NTPase and helicase activities (Schwer and Meszaros, 2000). However, the SAT motif does exhibit some

variation among different enzymes (TAT, FAT) which may confer functional specificity related to nucleic acid unwinding.

The only other conserved amino acid motif that has been characterized to date is the H/QxxGRxxR motif.   Interestingly, the DEAD subfamily possesses a HRIGR motif while the other two subfamilies possess a more flexible QxxGRxGR motif (Gorbalenya *et al.*, 1989; Linder *et al.*, 1989). In the DEAD box protein eIF-4A, the HRIGR motif was found to be strongly concerned with binding RNA and possibly in coupling ATPase, RNA binding and RNA unwinding activities (Pause *et al.*, 1993). However, the QxxGRxGR motif of the prototypal DExH RNA helicase, NPH-II of vaccinia virus, was shown by Gross and Shuman (Gross and Shuman, 1996) not to be involved in RNA binding at all. Instead, mutations to this motif resulted in defects in RNA unwinding and ATPase activity. It is a possibility that this motif compensates for a decrease in helicase activity that results from the presence of a histidine instead of an aspartate in the last position of the Walker B motif (Pause and Sonenberg, 1992; Walker *et al.*, 1982) in the DEAH or DExH subfamilies. Determining the functions of the remaining conserved motifs will also be a useful tool in understanding the differences between subfamilies of RNA helicases. However, it should be pointed out that the possession of conserved SF2 motifs does not guarantee helicase activity, nor does it have any clear predictive value in terms of template preference (DNA or RNA) or directionality of unwinding (3' to 5' or 5' to 3'). Despite these shortcomings however, the recent elucidation of the crystal structure of the hepatitis C virus (HCV) RNA helicase and subsequent modeling of an RNA

unwinding mechanism by Cho *et al.* (1998) has confirmed and further explained many of the results previously found through mutational analysis.

HCV RNA helicase is a member of the DExH subfamily and is required for replication of the positive strand RNA genome of the Hepatitis C virus (Jin and Peterson, 1995). Crystal structure analysis has determined that this enzyme consists of three domains, an NTPase domain, an RNA binding domain and a helical domain (Cho *et al.*, 1998). Confirmation that the Walker motifs (GKS and DECH) are involved in NTP binding and hydrolysis (Kim *et al.*, 1997) is seen through their lining of the active site of the NTPase domain (Cho *et al.*, 1998). Work by Cho *et al.* (1998) showed that the RNA binding domain and the NTPase domain are separated by an interdomain cleft made up of two loop structures that is large enough to hold a single strand of RNA. Supporting this theory is the revelation that the most conserved residues of the RNA binding motif, **QR**RG**R**TG**R**GRRG, point towards this interdomain cleft. The majority of the residues that comprise the RNA binding motif are located on a loop structure that could change its conformation thus favouring contact between the RNA phosphate backbone and the numerous arginine residues in the binding motif. One of the interdomain connecting loops contains the TAT motif which strengthens the initial idea that this motif is involved in coupling NTP hydrolysis to RNA unwinding. In fact, it is possible that the histidine residue from DECH functions as a triad with the threonine residues from TAT in this coupling process. Although the oligomeric state of the HCV RNA helicase is unconfirmed, a realistic dimeric form of HCV RNA helicase has been suggested, through

computer generated models, that forms a helical channel between the two molecules, which is surrounded by RNA binding domain residues. This model has led to a possible mechanism for RNA unwinding in a 3' to 5' directional manner.

It has been suggested that the presence of ssRNA in the interdomain cleft of one of the helicase molecules would induce a conformational change in the protein, thereby increasing the NTPase activity in the NTPase domain. Hydrolysis of the NTP would then result in dissociation of RNA from the cleft of the "active" molecule and subsequent bending motion and transformation of the dimer to a resting conformation. Rotation of the dimer could then occur, bringing the previously resting molecule into close contact with the RNA. Binding of the RNA by the interdomain cleft of this helicase molecule would then allow a repeated cycle of NTP hydrolysis and subsequent release and relaxation into the resting conformation to occur (Fig. 1.4). It is this sequence of events that comprise the proposed "molecular see-saw" theory that results in RNA unwinding with one strand of the RNA being passed through the channel while the second strand is separated and left hanging out of the dimer.

Because both the sequence and spacing of the characteristic helicase amino acid motifs are conserved, it is attractive at this point to idealize that all or most RNA helicase structures will be similar and that the model for unwinding RNA duplexes using these enzymes will be comparable. However, despite these known and assumed similarities in sequence and structure, it is clear that the

Figure 1.4: Schematic drawing of the proposed mechanism of RNA unwinding by Hepatitis C Virus (HCV) RNA helicase. (A) An RNA helicase (shown in dimeric form) binds to a single stranded end of an RNA duplex. (B) The single stranded RNA is bound by the interdomain cleft of one molecule (shown in purple). Binding is proposed to induce a conformational change in the molecule, which increases NTP hydrolysis. (C) NTP hydrolysis in B causes dissociation of the RNA from the interdomain cleft of the molecule and subsequently results in a rigid body rotation along the RNA strand which stabilizes the dimer through contact with the RNA binding motifs. The helicase dimer translocates along the single stranded RNA and the interdomain cleft of the second helicase molecule (shown in green) binds the RNA. (D) The RNA helicase dimer reaches the junction between single stranded and double stranded RNA after repeated cycles of translocation described above. (E) In the same manner as above, the dimer continues to translocate along the same strand of RNA. The base pairs are disrupted using energy supplied by favourable interactions between the interdomain cleft and the single stranded RNA. The double stranded RNA is unwound as one strand is passed through the channel at the dimer interface while the other strand is left hanging out of the dimer.

(Modified from Cho *et al.*, 1998)

A

B

C

D

E
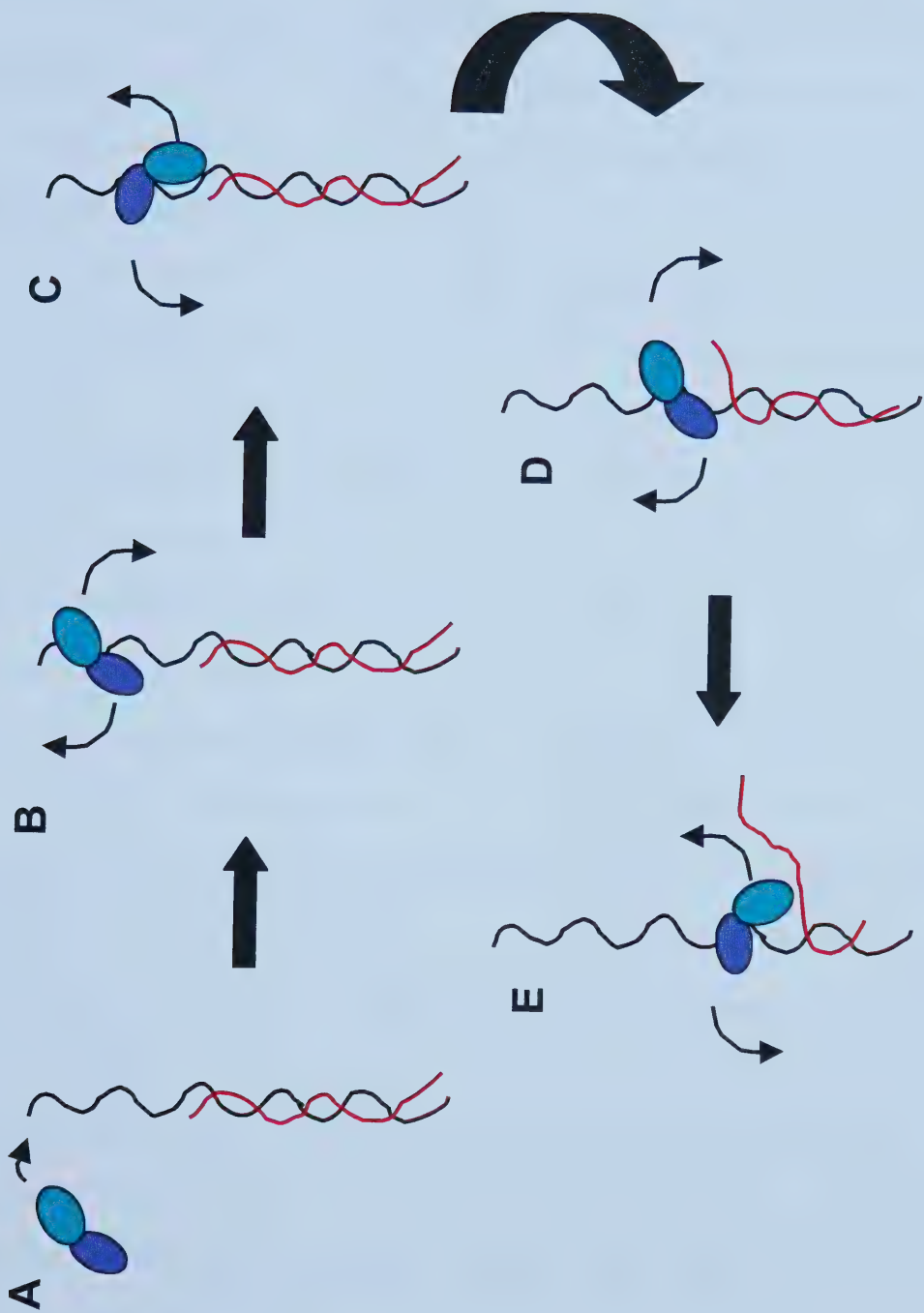
functions of individual RNA helicase enzymes are very different, even within the enzyme subfamilies.

The most characterized of the RNA helicases is the ubiquitous enzyme eIF-4A. A member of the DEAD subfamily, this enzyme plays a pivotal role in translation initiation in eukaryotes by unwinding secondary structure in the 5' untranslated region of mRNA transcripts (Rozen *et al.*, 1990). This linearization of the mRNA allows for the 40S ribosomal subunit to bind its template and begin translation. eIF-4A-like enzymes have been identified in nearly all eukaryotes, lending credibility to its global importance in translating eukaryotic mRNA. Also a member of the DEAD subfamily are the cold shock RNA helicases which were first identified in *E. coli* (CsdA; Jones *et al.*, 1996) but have since also been identified in multicellular cyanobacteria *Anabaena* sp. (CrhC; Chamot *et al.*, 1999). CsdA (for cold shock DEAD-box protein A) is expressed upon a shift-down in temperature and is believed to be associated with the ribosome during cold shock adaptation (Jones *et al.*, 1996). Secondary structures that would impede translation of mRNA by ribosomes are presumably more stable at the lower temperatures associated with cold shock and thus a helicase is thought to be required to unwind these structures in order for efficient translation to occur. In reality, CsdA is most likely just one member of a proposed multisubunit complex required for gene expression during cold shock (Mitta *et al.*, 1997; reviewed in Sommerville, 1999). This complex includes not only a ribosome and CsdA, but also the major cold shock protein of *E. coli*, CspA, which likely

associates with the unwound RNA to prevent the secondary structure from simply reforming.

The association of a single-stranded RNA-binding protein complementing the action of an RNA helicase is also found in the *E. coli* "degradeosome". In this ribonucleoprotein complex, the DEAD-box RNA helicase RhlB is necessary for efficient degradation of stem-loop structures by polynucleotide phosphorylase (PNPase), a processive 3' to 5' exoribonuclease (Coburn *et al.*, 1999; Py *et al.*, 1996). This situation mirrors the interaction of DExH proteins, such as Ski2, with *S. cerevisiae* exosomes where mRNA turnover has been shown to be essential for viability (Jacobs *et al.*, 1998). Interestingly, it appears that RhlB may also be essential in *E. coli* as attempts to obtain a true deletion in the *rhlB* gene have been unsuccessful (cited in Py *et al.*, 1996).

In the yeast *S. cerevisiae* the spliceosome is composed of several proteins with RNA helicase activity required for specific stages in mRNA splicing. Eukaryotic pre-mRNA's are spliced via two successive transesterification reactions. The first step results in a 2' – 5' branched lariat intermediate with a 3'-OH – terminated exon. This resulting 3'-OH attacks the phosphodiester bond at the 3' splice site for the second processing step, which results in exon ligation and expulsion of the lariat intermediate (reviewed in Schwer, 2001). The DEAH RNA helicase Prp22p is an important enzyme in splicing as it plays two distinct roles in the process. Its first role is an ATP-independent one during the second step of RNA splicing (McPheeters *et al.*, 2000; Schwer and Gross, 1998), while its second purpose is to utilize ATP during spliceosome disassembly. Prp22p is

one RNA helicase in which it has been demonstrated that the NTPase and helicase activities are not obligately coupled, thus discounting the conjecture that all RNA helicases are ATP-dependent RNA unwinding enzymes (Schwer and Meszaros, 2000).

In addition to the widespread consequences of their function in translation initiation and mRNA processing, RNA helicases have also been implicated as key regulators of development and differentiation. In *Drosophila*, for example, the DEAD RNA helicase, *vasa*, is an enzyme involved in regulating pole plasm assembly and function and for completion of oogenesis (Liang *et al.*, 1994). This helicase has been shown to regulate spatial expression and localization of mRNA transcripts required for proper body plan development (Gavis *et al.*, 1996; Styhler *et al.*, 1998; Webster *et al.*, 1997). Vasa orthologues have been isolated in numerous species including humans (Castrillon *et al.*, 2000), solidifying its function as a developmental regulator. Also in *Drosophila*, the DExH protein encoded by the *maleless* (*mle*) gene is indispensable for male viability through proper dosage compensation (Lee *et al.*, 1997). Results imply that Mle is required to enhance transcription of genes on the male X chromosome to a level comparable to that of XX females. Mutations in this gene are lethal, stressing again the developmental importance of this DExH helicase.

Recently, a new developmentally significant DExH RNA helicase has been identified in *Arabidopsis thaliana*. *caf* encodes a protein that helps specify determinate growth in floral meristems and is an apparent suppressor of cell division in these tissues (Jacobsen *et al.*, 1999). Mutants in *caf* display various

developmental defects including unregulated cell division in the center of the flower. Of great interest is the suggestion that the Caf enzyme may act in a different manner depending on which plant tissue it is expressed in. Therefore, localization of developmental signals is an important requirement for proper differentiation in which the putative RNA helicase, Caf, plays a significant part.

As can be inferred from the ubiquitous presence of RNA helicases, the possibility of one or more of these enzymes being present in *Streptomyces* is high. During the initial cloning and sequencing of the *bldG* locus of *S. coelicolor* (Bignell *et al.*, 2000), a partial open reading frame (ORF) was identified that was transcribed divergently from *bldG* itself, and which showed striking similarity to numerous RNA helicases including the well-characterized eIF-4A. Searches of the *S. coelicolor* genome sequencing project database (http://www.sanger.ac.uk/ Projects/S_coelicolor/) enabled the identification of a 2.4 kb ORF that has been designated SCH5.13 (Fig. 1.5) and whose deduced amino acid sequence identifies it as a member of the DExH subfamily of RNA helicases (Fig.1.6). The conserved nature of helicase amino acid sequences increases the possibility that SCH5.13 is a putative RNA helicase enzyme. So far the genome sequencing project database (see above) has identified thirteen putative helicase-encoding genes, 3 of which appear to be members of the DExH subfamily. Given that these proteins have been implicated in such a wide variety of cellular processes, including differentiation, it is exciting to imagine that SCH5.13 could be an essential component to *S. coelicolor* differentiation processes. This, together with the ORF being transcribed divergently from *bldG*, a key regulatory element

Figure 1.5: Cosmid map of *S. coelicolor* genome. Taken from Redenbach *et al.* (1996). The approximate location of the putative RNA helicase, SCH5.13, is indicated by the black arrow with an asterisk.

Row 1
1C9 · J4 · J21 · J1 · J9A · F1 · SG9 · F41 · F51 · F76 · F34
LHE · J30 · J11 · J33 · J12 · F20 · F8S · F12 · F62 · F51A · F90 · F6
AseI-J | AseI-F

Row 2
F6 · F73 · SG5 · FS6 · F1S · 3/SB7 · 3/F60 · F43A · M1 · M11 · 2/G2 · G22
F11 · 8B3 · F5S · F9I · F42 · F8I · F43 · M1 · M10 · BAC19F3 · G20A
AseI-F | AseI-M | AseI-G

Row 3
G22 · 2/G38 · 2/G11 · 2/G5S · 2/G18 · 2/G6I · 1A8A · 6D7A · 9C5 · L11
2/G4 · G8A · G11A · 2/G1 · BAC36FS · 10A9 · 6D7 · L6 · L2 · L24
AseI-G | AseI-L

Row 4
I35 · I46 · I30A · 2/I34 · I5 · I28 · I39 · C22 · 3C9 · 3A3 · 4A10
L24 · I41 · I52 · I11 · I51 · I33 · I8 · I7 · CS4 · 7H2 · 4G6 · 2C1A
AseI-L | AseI-I | AseI-C

Row 5
6E10 · 6G10A · 3H12 · BAC17D6 · C75A · CS3 · 1E12 · 8A2 · C34 · C82
2C1A · 6G10 · SF7 · 10B7 · 1G2 · C30 · C8A · 4A7 · C42 · 7A8 · C121
AseI-C

Row 6
C117 · C123 · C80 · 8E4A · C61A · CS7A · 2/C13 · E20 · E19A · E49
C121 · C77 · C88 · 8E4 · 6D10 · C46 · C10S · BAC17F8 · E6 · E56 · E50
AseI-C | AseI-E

Row 7
E99 · E34 · E25 · E66 · E22 · E63 · E39 · E68 · E94 · E9 · E46
E50 · E33 · BAC19G2 · E41 · E87 · E8 · E29 · E15 · E7 · E126 · E36 · E65
AseI-E

Row 8
E65 · E2 · H17 · 66T3 · H44 · H22A · AC2 · H69 · H24 · D7B · 2/10A7
E134 · H5 · H66 · H10 · H35 · H63 · GD3 · H18 · Q11 · BAC2SE3
AseI-E | AseI-H | AseI-Q | AseI-D

Row 9
2/10A7 · D25 · D17A · D84 · 2D46 · D49 · D95A · D19 · D10 · D6 · D69
2/D60 · D17 · D72A · D66 · D8A · D66A · D12A · 2/D82 · 6F11 · D6S · D3S
AseI-D

Row 10
D3S · D16A · D39 · D40A · 6G4 · D63A · SG8 · 2/K8 · K13 · K15
2/D4 · D20 · D82 · D31 · D63 · 2A6 · K20 · 2/K31 · 2/K36 · K7
AseI-D | AseI-O | AseI-K

Row 11
BAC20F6 · BAC31E11 · P8 · 7E4 · CB12 · BACSH2 · 8F4 · 3D11 · 8D9 · 7A1 · 6A9
K7 · BAC28G1 · 9E12 · 2/3B6 · 2/7G11 · 6G9 · 2/6GS · 6A11 · 2A11 · 1C2 · 2E1 · 8B7
AseI-K | AseI-N | AseI-P | AseI-B

Row 12
8B7 · 9F2 · 9A10 · 4H8 · S8B · 2E9 · 10A5 · BAC16H6 · 7B7 · 1B5 · BAC1A6 · 9B2
SH4 · 3C3 · 7C7 · 4H2 · 9B10 · 3F7 · 7H1 · BAC1C11 · 1C3 · 9B1 · BAC33E8 · 1A9
AseI-B

Row 13
6C5 · 9G1 · AH10 · 1G7 · IF3 · 3A7 · 3C8 · 9B5 · 9C7 · SC7 · 3F9 · 1F2 · 5A7
1A9 · 2G5 · 2H4 · 2C4 · BAC8D1 · 10R5 · 4A2 · LA6 · 1A11 · 1E6 · 4B5 · 8A6 · 4G2 · 6G3
AseI-B | AseI-A

Row 14
6G3 · SF2A · 1A2 · 3D9 · 1B2 · 6F7 · 1H10 · 4G1 · 4B10
4C6 · 6AS · 4A9 · 7P9 · 1G8 · 8F11 · 2C3 · 3A4 · 8B1
AseI-A

Row 15
8B1 · 8A11 · 2H12 · SH1 · 4G10 · 10G8 · SC11 · BAC17A6 · 8G12 · 7H9 · 10F4
9A4 · 1D2 · 7A12 · SF8 · 9H11 · 6D11 · BAC14E8 · BAC2SF8 · SF1 · 2H2 · 4C2
AseI-A

Row 16
4C2 · BAC12C8 · SE9 · 8E7 · END
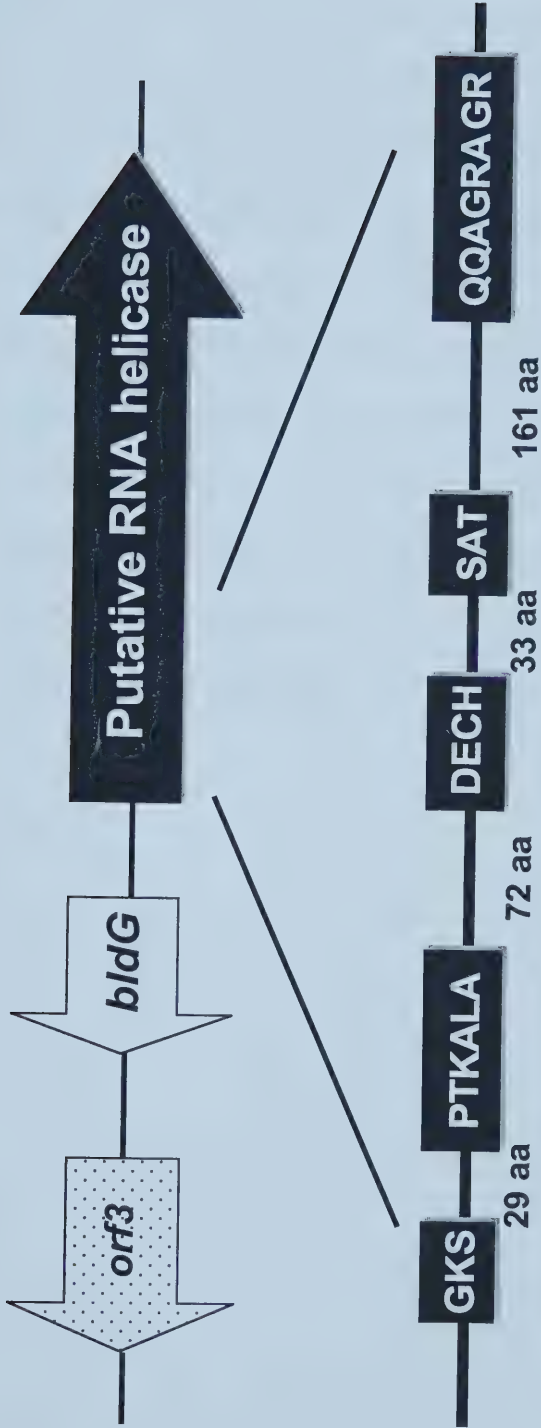1A4 · 6D11 · 10B8A · J24
AseI-A

Figure 1.6: Schematic drawing of the *bldG* locus of *Streptomyces coelicolor.* Included are the conserved amino acid motifs found in the putative RNA helicase, SCH5.13, and the amino acid spacing between them: Drawing not to scale.

in *S. coelicolor* development, has made SCH5.13 an exciting subject for study. The main objective of this thesis was to examine whether SCH5.13 is involved in *S. coelicolor* development. This was achieved through analysis of the expression pattern of SCH5.13 to ascertain if it was transcribed in a growth phase dependent manner. Mapping the transcription start site of this ORF was also considered important in deciding whether expression of *bldG* and SCH5.13 is coordinately controlled. Furthermore, determining the point of transcription initiation was helpful in concluding whether the translation start codon predicted by the genome sequencing project is in fact correct, despite the lack of both a reasonable ribosome binding site and an easily identifiable promoter. Finally, attempts to create a null mutant of the SCH5.13 ORF have been useful in elucidating what role, if any, the putative RNA helicase could play in *S. coelicolor* development.

Chapter 2:

Materials and Methods

2. MATERIALS AND METHODS

2.1 Bacterial Strains, Plasmids and Growth Conditions

2.1.1 *Streptomyces* and *E. coli* strains

All *E. coli* strains used in this study are listed in Table 2.1. All *Streptomyces* strains used in this study are listed in Table 2.2.

2.1.2 Cloning vectors used in this study

All cloning vectors used in this study are listed in Table 2.3.

2.1.3 Recombinant plasmids constructed and used

All recombinant plasmids, cloning strategies and their uses are listed in Table 2.4.

2.1.4 Growth and maintenance of *E. coli* strains

Liquid *E. coli* cultures were grown in LB medium at 37°C on a rotating rack. Solid cultures were grown on LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) with 1.5% agar (w/v) at 37°C. When necessary, ampicillin was added to 100 μg/mL or apramycin added to 50 μg/mL. *E. coli* strains were maintained as frozen (-86°C) stocks in 20% glycerol and as solid cultures at 4°C on LB medium with 1.5% agar (w/v).

Table 2.1: *Escherichia coli* strains

E. coli strain	Genotype	Reference or Source
DH5α	F, φ80*lacZΔM15Δ(lacZYA-argF)U169, deoR,recA1,hsdR17(r$_K$-m$_K$-) phoA, supE44, λ-, thi-1, gyrA96, relA1*	[Hanahan, 1983 #1138]; Gibco BRL
ET12567	F'dam13::Tn9, dcm6, hsdR, recF143, zij202:: TN10, galK2, galT22, ara14, lacY1, xyl5, leuB6, thi1, tonA31, rpsL136, hisG4, tsx78, mtl1, glnV44	[MacNeil, 1992 #188]; gift from D. MacNeil, Merck Sharp and Dohme Research Laboratories

Table 2.2: *Streptomyces* Strains

Streptomyces strain	Genotype	Reference or Source
Streptomyces coelicolor A3(2) J1501	hisA1, uraA1, strA1, pgl, SCP1⁻, SCP2⁻	[Chater, 1982 #59]; John Innes Institute
Streptomyces lividans 66 1326	SLP2, SLP3	[Lomovskaya, 1980 #972];John Innes Institute

Table 2.3: Cloning vectors used in this study

Plasmid	Antibiotic Marker	Relevant Characteristics	Reference or Source
E. coli plasmids, phagemids & shuttle vectors			
pBluescript® KS	Ampicillin	High copy number phagemid containing T7 and T3 polymerase promoters.	[Alting-Mees, 1989 #1139]; Stratagene
pSET152	Apramycin	High copy cloning vector in *E. coli*, integrates into *Streptomyces* φc31 *att* site.	[Bierman, 1992 #139]; Northern Regional Research Center, Peoria, Ill.
pAU190	Apramycin	pSET152 with promoterless *xylE* gene in *BamH1* site.	Gift from Marie Elliot
pUC119	Ampicillin	High copy number phagemid cloning vector.	[Vieira, 1987 #140]; J Vieira, Waksman Institute of Microbiology, Piscataway, N.J.
pUC119ApR	Ampicillin	pUC119 with Apramycin resistance gene in *BamHI – EcoRI* in polylinker.	[Bignell, 2000 #1052]
pUC120	Ampicillin	High copy number phagemid vector with NcoI site.	[Vieira, 1987 #140]; gift from Annie Wong
pIJ2925	Ampicillin	pUC18 derivative with polylinker flanked by *BglII* sites.	[Janssen, 1993 #62]
pAU5	Ampicillin	pIJ2925 with *tsr* marker.	[Giebelhaus, 1996 #634]
Streptomyces plasmids			
pIJ4083	Thiostrepton	High copy number promoter probe vector containing promoterless *xylE*.	[Clayton, 1990 #366]; John Innes Institute

Table 2.4: Recombinant plasmids constructed and used in this study

Plasmid	Parent Plasmid	Selective Markers	Insert size	Cloning Strategy	Use
pAU220	pBluescript® KS	Ampicillin	740 bp	Insert fragment with *Hind*III site 186 nucleotides upstream of helicase start codon and *Xba*I site 553 nucleotides downstream of start codon.	Riboprobe construction
pAU221	pAU230	Apramycin	1300 bp	Promoterless *xylE* in *Bam*HI site downstream of helicase promoter.	Single copy helicase promoter probe.
pAU222	pIJ4083	Thiostrepton	240 bp	*Bam*H1 fragment containing helicase promoter in *Bam*HI site.	Multi-copy helicase promoter probe.
pAU224	pAU231	Apramycin	1300 bp	Promoterless *xylE* in *Bam*HI site downstream of *bldG* promoter.	Single copy *bldG* promoter probe.
pAU225	pIJ2925	Ampicillin	690 bp	Internal helicase fragment from *Bam*HI site 443 nucleotides downstream of start codon to *Kpn*I site 1137 nucleotides downstream; ends were blunt-ended and ligated into the *Hind*III site that had been blunt-ended.	Helicase gene disruption.
pAU226	pAU225	Ampicillin	1000 bp	*Xba*I/*Sma*I *tsr* gene inserted in polylinker.	Helicase gene disruption.
PAU227	pUC120	Ampicillin	3140 bp	Entire helicase gene from *Sac*I site 366 nucleotides upstream of start codon to *Nco*I gene 352 nucleotides downstream of stop codon. *Sac*I / *Nco*I fragment cloned directly into the same sites in polylinker.	Complementation experiments and double helicase mutant disruption.

Plasmid	Parent Plasmid	Selective Markers	Insert size	Cloning Strategy	Use
pAU228	pSET152	Apramycin	3200 bp	Insert DNA excised from pAU227 using NcoI and XbaI. NcoI site blunt-ended and the DNA was cloned into XbaI / EcoRV sites of parent vector.	Complementation experiments and double helicase mutant disruption.
pAU229	pAU225	Ampicillin	1300 bp	BamH1/EcoR1 apramycin resistance gene.	Disruption of helicase in bldG3b mutant.
pAU230	pSET152	Apramycin	240 bp	Blunt-ended fragment containing helicase promoter region (226 nucleotides upstream to 12 nucleotides downstream) in blunt-ended EcoRI site.	Helicase single copy promoter probe.
pAU231	pSET152	Apramycin	240 bp	Insert DNA identical to that of pAU230 except that the promoter is in the opposite orientation.	bldG single copy promoter probe.
pAU233	pIJ4083	Thiostrepton	240 bp	Insert DNA identical to that of pAU222 except that the promoter is in the opposite orientation.	bldG multi-copy promoter probe.

2.1.5 Preparation of *E. coli* glycerol stocks

E. coli cultures were grown overnight in LB medium on a rotating rack at 37°C. The resulting cultures were then separately mixed with glycerol to a final concentration of 20% glycerol and flash frozen on dry ice. The frozen stocks were stored at −86°C.

2.1.6 Growth and maintenance of *Streptomyces* strains

Five milliliter liquid cultures were grown where specified either in Trypticase Soy Broth (TSB; 30 g/L) or R2YE (Hopwood *et al.*, 1985) broth at 30°C, with shaking, in 20 mL universal vials with a one inch spring coil to disperse the mycelia. Twenty-five milliliter cultures were grown, where specified, in either a 3:2 "super" YEME (3 g/L yeast extract, 5 g/L peptone, 3 g/L malt extract, 55 mM glucose, 1 M sucrose, 5 mM $MgCl_2$, 0.5% glycine, tiger milk (Hopwood *et al.*, 1985)):TSB mixture or R2YE broth with shaking at 30°C in a 250 mL flask with a spring coil.

Surface grown cultures were grown on cellophane discs on R2YE agar at 30°C. Cultures grown specifically for spore stocks were grown on SpMR-Mtl (5 g/L Mannitol, 1 g/L Yeast Extract, 10 g/L $MgCl_2 \cdot 6H_2O$, 4.2 g/L MOPS, Trace Elements, 103 g/L Sucrose, Tiger Milk, 20 mM $CaCl_2$) (Kendrick and Ensign, 1983) with 2.2% agar (w/v) at 30°C.

Where necessary for both solid and liquid cultures, thiostrepton was added to either 5µg/mL or 50µg/mL (as indicated). Apramycin, when required,

was added to 50 µg/mL. *Streptomyces* strains were maintained as frozen (-20˚C) glycerol stocks of spores and as solid cultures at 4˚C on R2YE agar plates.

2.1.7 Preparation of *Streptomyces* spore stocks

Fully sporulated plate cultures were scraped with a spatula into sterile universal vials with 5-10 mL H_2O and vortexed for 30 seconds. The homogenate was then placed in a sonication bath for 10 to 15 minutes to release the spores from the mycelia, after which cultures were placed on ice to allow the vegetative biomass to settle. The top liquid layer was filtered through sterile cotton and the filter was rinsed with 1-2 mL H_2O. The filtrate was collected in sterile universal vials and was centrifuged for 10 minutes at 2000 rpm in an International Centrifuge Refrigerated Model PR-J, after which the supernatant was discarded. The pellet was resuspended in the remaining liquid and glycerol was added to a final concentration of 20% (v/v). Spores were aliquotted into sterile bijou bottles and stored at –20˚C.

2.2 DNA Isolation and Transformation

2.2.1 Preparation of *E. coli* competent cells

A single colony of a desired *E. coli* strain was used to inoculate 2 mL of LB medium. The culture was grown overnight at 37˚C in a stationary rack. The resulting culture was diluted 1:100 and grown for 3-4 hours at 37˚and 250 rpm. The culture was centrifuged in sterile universal vials for 5 minutes at 2000 rpm See Sec. 2.1.7). The pellet was washed with 5 mL of 100 mM $CaCl_2$ and re-

centrifuged as before. This washing step was repeated a second time. The

pellet was resuspended in 100 mM $CaCl_2$ and 20% glycerol to a final volume of 1

mL. Aliquots of 50 – 100 µL were flash frozen on

dry ice and stored at –86˚C.

2.2.2 Transformation of *E. coli* strains

Commercially obtained *E. coli* DH5α was transformed according to

manufacturer's instructions (Gibco BRL). Transformations using lab stocks of *E.*

coli DH5α and ET12567 were as for the commercial cells except that heat

shocking was carried out at 42˚C for 70 seconds.

2.2.3 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* cultures using alkali lysis as

described in Sambrook *et al.* (Sambrook *et al.*, 1989). For large scale

preparations (25 mL or 500 mL) solution volumes were scaled up accordingly.

2.2.4 Preparation of *Streptomyces* protoplasts

Protoplasts were prepared as described in Hopwood *et al.* (1985).

2.2.5 Transformations using *Streptomyces* strains

All transformations in *Streptomyces* were performed as described in

Hopwood *et al.* (Hopwood *et al.*, 1985).

2.2.6 Isolation of plasmid DNA from *Streptomyces*

Plasmid DNA was isolated from *Streptomyces* cultures using modified alkali lysis as described in Hopwood *et al.* (Hopwood *et al.*, 1985). For large-scale preparations (25 mL, 250 mL, 500 mL), solution volumes were scaled up accordingly.

DNA isolated from a large-scale culture was purified via CsCl isopycnic sedimentation. The DNA pellet was dissolved in 8mL TE buffer (10 mM Tris, 1mM EDTA), pH 8. Cesium chloride and ethidium bromide were added to final concentrations of 1 mg/mL and 0.1 mg/mL, respectively. The final mixture was centrifuged for 40 hours in a Beckman LB-M Ultracentrifuge at 35 000 rpm using a 50-Ti rotor. After centrifugation, the band corresponding to covalently closed circular plasmid DNA was extracted and dialyzed against TE buffer. Finally the DNA was precipitated and redissolved in TE buffer to a final concentration of 1 µg/mL.

Isolation of plasmid DNA from *Streptomyces* plate cultures was as described in Soliveri *et al.* (1999).

2.2.7 Isolation of chromosomal DNA from *Streptomyces*

Chromosomal DNA was isolated from *Streptomyces* liquid cultures by Procedure 3 as described in Hopwood *et al.* (Hopwood *et al.*, 1985). When indicated, chromosomal DNA was isolated from plate cultures. Cultures were scraped into homogenizer tubes with 5 mL of sterile H_2O and crushed prior to

the first centrifugation step in Procedure 3. The remainder of the procedure was then carried out as described.

2.3 DNA Analysis and Purification

2.3.1 Digestion and cloning of DNA

Restriction endonuclease digestions of both plasmid and chromosomal DNA were carried out as advised by the manufacturer (Roche or New England Biolabs), and as in Sambrook et al. (Sambrook et al., 1989). When necessary, DNA harbouring cohesive overhangs was blunt-ended using 1 U of Klenow enzyme (Roche) in a 30 µL reaction mixture. The reaction mixture contained 5mM Tris, pH7.8, 0.5 mM $MgCl_2$, 1 mM β-mercaptoethanol and 5 µg/mL BSA, and was incubated for 8 minutes at 37°C before adding 2 µL of 0.125 mM dNTP's (dATP, dTTP, dGTP, dCTP) and incubating for a further 10 minutes at 37°C. Ligations were executed using an insert to vector ratio of ~3:1 for sticky-end ligations and ~10:1 for directional ligations and blunt ligations. Ligation mixtures were incubated at 15°C for at least 8 hours for sticky-end ligations or at room temperature for 4 hours for blunt-end ligations. Directional ligations with blunt and cohesive ends were first incubated at room temperature for 3 hours and then transferred to 15°C water bath overnight. All reaction mixtures utilized 1 mM ATP, 0.5 U T4 DNA ligase (Roche) in a buffer of 50 mM Tris-HCl (pH7.6), 10 mM $MgCl_2$, 5% PEG-8000, and 1 mM DTT.

2.3.2 Polymerase chain reaction

Polymerase chain reaction was used to amplify DNA fragments used in cloning, as well as probes used for northern and Southern hybridization analysis and S1 nuclease mapping. PCR was also used to screen for cloned DNA in various plasmids and to amplify cDNA for reverse transcriptase polymerase chain reaction (RT-PCR) analysis. All oligonucleotide primers and their specific uses are listed in Table 2.5.

For cloning, probe construction and specified screening purposes, amplification reactions were performed in 100 µL volumes in 0.6 µL tubes (Rose Scientific). These reactions utilized the EXPAND™ High-Fidelity Polymerase kit (Roche) and were amplified in the Techne PCH-2 Thermocycler. Typical reaction mixtures contained 1 mM $MgCl_2$, 1 X Buffer 2 (Roche), 0.2 mM dNTPs, 40 pmol of each oligonucleotide primer, 4% DMSO and 2.5 U of Expand polymerase. All reactions contained 1µg of chromosomal DNA or 10 ng plasmid DNA as template.

For amplification of cDNA for RT-PCR, reactions were carried out in 50 µL volumes in 0.2 mL tubes (Rose Scientific) in the MJ Research Minicycler™, using the EXPAND™ system. Reaction mixtures contained 0.25 mM dNTPs, 1.2 X Buffer 3 (Roche), 0.75 M Betain, 18 pmol ^{32}P- labeled primer, 18 pmol unlabeled primer, and 2.63 U Expand polymerase.

Amplification for screening of cloned DNA utilized Taq polymerase (gift from M.A. Pickard, University of Alberta) in 20 µL reaction volumes in 0.6 mL tubes (Rose Scientific) using either the Techne thermocycler or the MJ

Table 2.5: Oligonucleotide Primers*

Primer	Sequence (5' – 3')	Region of Homology	Use
BKL87	GTGCCGGTGGCGACGAC	SCH5.13 nucleotides +207 – +223 **.	PCR for Northern probe, RT-PCR, sequencing.
BKL88	ATGCTCCTGGACCGGCTC	SCH5.13 nucleotides +1 – +18.	PCR for Northern probe, RT-PCR.
BKL80	CGCGCTGCAGGCTCACGCAGCTTGGGC	Upstream of SCH5.13 nucleotides -323 – -306, includes *Pst*I site.	PCR for S1 Mapping probe.
BKL82	CGCGTCTAGACGACGTACTTCAGTGCC	SCH5.13 nucleotides +556 – +539, includes *Xba*I site.	PCR for riboprobe cloning.
BKL91	CGGCAAGCTTGACACCTCGGCTTGAAG	Upstream of SCH5.13 nucleotides -187 – -170, includes *Hind*III site.	PCR for riboprobe cloning.
JWA0	CCAGGGATGTTCGATGC	SCH5.13 nucleotides +159 – +143.	primer extension, sequencing.
DBG6	GCCAAGAAGACCACCGCCAAG	*hrdB* DNA sequence.	RT-PCR control.
DBG7	GACCTTGCCGATCTGCTTGAG	*hrdB* DNA sequence.	RT-PCR control.
JWA4	GACACCTCGGCTTGAAG	Same as BKL91 without a restriction site included.	RT-PCR
JST1	CGCGGGATCCCGGTCCAGGAGCATGC	SCH5.13 nucleotides +15 – -3, includes *Bam*H1 site.	RT-PCR, PCR of promoter region for cloning.
JST4	CGATCCCGGCGGGAGCCCGGCT	Upstream of SCH5.13 nucleotides -39 – -18.	RT-PCR
JST5	CGCCTTGGTCCCATTGTCCGTCA	Upstream of SCH5.13 nucleotides -109 – -87.	RT-PCR

Primer	Sequence (5' – 3')	Region of Homology	Use
JST6	CACTTACCGGCAGGCGT	Upstream of SCH5.13 nucleotides ⁻132 – ⁻117.	RT-PCR
JWA19	GCTCACGCAGCTTGGGC	Identical to BKL80 with no restriction site included.	PCR of promoter region for cloning.
JWA5	CACCGAGTCTGTCACTG	Upstream of SCH5.13 nucleotides ⁻67 – ⁻83.	Sequencing for promoter orientation in pAU222.
BKL67	ACCTCTCGGAACCTCCG	SCH5.13 nucleotides +380 – +397.	PCR of internal fragment for cloning.
JST2	GCCTGCTGCCACAGCGAG	SCH5.13 nucleotides +1172 – +1155.	PCR of internal fragment for cloning.
JST7	CGCCAGGGTTTCCCAGTCACGAC	pSET152 flanking polylinker.	PCR of insert for promoter probe experiments.
JST8	GAGCGGATAACAATTTCACACAGGA	pSET152 flanking polylinker.	PCR of insert for promoter probe experiments.
JST9	AACCGATACAATTAAAGGCT	terminator of phage (tfd) region of pIJ4083.	PCR of insert for promoter probe experiments.
JST10	GTTCCAGGGCCTTGCTCATGT	5' end of xylE from pIJ4083.	PCR of insert for promoter probe experiments.

*Restriction endonuclease sites underlined.
** Nucleotide designations given with respect to SCH5.13 proposed ATG translation start codon.

Minicycler. Typical reaction mixtures contained 2 μL DNA sample (isolated as in 2.2.6) 3.6 pmol of each primer, 4% DMSO, 0.18 mM dNTPs, and 1.2 U Taq polymerase.

All amplifications using the Techne thermocycler were overlaid with mineral oil, reactions using the MJ Minicycler did not require mineral oil as a heated lid was used. All reactions were subjected to a 5 minute denaturation at 95°C, followed by 30 cycles of a 30 second denaturation at 95°C, 30 second primer annealing at 52°C and 1 minute extension at 68°C. All exceptions are as indicated in results.

2.3.3 DNA analysis using agarose gel electrophoresis

DNA fragments between 0.5 and 10 kb were electrophoresed at 100 volts on a 1% agarose, 1 X TBE (90 mM Tris, 89 mM Boric Acid and 2.5 mM Na_2EDTA) gel. Lambda DNA digested with *Pst*I was used as a DNA molecular weight marker in most cases. For smaller DNA fragments (0.5 to 1 kb), 2% agarose, 1 X TBE gels were used and molecular weight marker V (Roche) provided a DNA molecular weight ladder. To each sample 1/10 volume loading dye (0.25% bromophenol blue, 40% sucrose) was added to visually assess DNA progression. Gels were stained in 1 X TBE containing ethidium bromide and DNA bands were visualized on a UV transilluminator.

2.3.4 Purification of DNA from agarose gels

The trough purification method described in Zhen and Swank (1993) was used to purify DNA fragments larger than 1 kb. Agarose gels [0.7 – 1.4%, 1X TAE (40 mM Tris-acetate, 1 mM EDTA)] were poured to 0.5 – 1 cm thick using flat plates with raised, taped edges to form the mold. Loading dye was added, as described above, to visualize DNA progression. Gels were electrophoresed in 1X TAE containing ethidium bromide at 86 – 107 volts (as indicated) until the dye had reached 2/3 length of the gel. After visualization under UV light, a trough was cut immediately following the DNA band of interest. The gel was replaced in the electrophoresis tank and buffer removed until it reached half way up the gel but did not enter the trough. The trough was filled with a solution of 15% PEG-8000 and 0.5 µg/mL ethidium bromide in 1 X TAE. Electrophoresis was carried out at ~146 volts. DNA migration into the trough was followed using a hand-held UV illuminator (Mineralight® Lamp UVSL-25; Ultra Violet Products Inc.). Once the DNA band of interest had migrated into the trough, electrophoresis was halted and the trough contents were removed. Phenol chloroform and chloroform extractions were performed and the sample was precipitated using 1/10 volume 3 M sodium acetate and 2 volumes 95% ethanol.

2.3.5 DNA analysis using polyacrylamide gel electrophoresis

DNA fragments of 0.1 – 1 kb were electrophoresed at 200 volts on a 5% polyacrylamide gel (29:1 acrylamide: N,N'-methylene bisacrylamide) using a 1 X TBE buffer system. Molecular Weight Marker V (Roche) was used to determine

DNA band length for fragments smaller than 0.5 kb, and for fragments larger than 0.5 kb, Molecular Weight Marker III (Roche) was used. DNA progression was followed using the same loading dye as described above. Gels were stained in 1 X TBE containing ethidium bromide and visualized using a UV transilluminator.

2.3.6 Purification of DNA after polyacrylamide gel electrophoresis

The crush and soak purification method (Sambrook *et al.*, 1989) was used to purify fragments smaller than 1 kb. DNA of interest was electrophoresed on a 5% polyacrylamide gel at 200 volts until the loading dye reached 2/3 length of the gel. The gel was stained in ethidium bromide as described above and DNA bands were visualized using a UV transilluminator. The DNA band of interest was excised from the gel using a scalpel and placed in a 1.6 mL tube (Fisher). The gel slice was crushed against the side of the tube and 1-2 volumes of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, pH 8.0) was added to the crushed gel. The tube was incubated overnight at 37˚C on a rotating rack. Following incubation, the acrylamide-DNA-containing mixture was centrifuged for 1 minute at 13 000 rpm to separate the gel from the buffer containing the DNA of interest. The supernatant was transferred to a separate 1.6 mL tube (Fisher). An additional 0.5 volume of elution buffer was added to the sedimented gel and the mixture was vortexed to wash the pellet. The mixture was then centrifuged as above and the supernatant added to the tube containing the first supernatant. To ensure that no polyacrylamide was remaining in the DNA mixture, the tube containing the pooled supernatants was centrifuged for 1 minute as above and

the supernatant removed to another 1.6 mL tube. Two volumes of 95% ethanol

and 1 µL of glycogen were added to the solution to precipitate the DNA. The

precipitated DNA was redissolved in 100 µL TE buffer and a second precipitation

was performed using 1/10 volume sodium acetate, 2 volumes of 95% ethanol,

and 1 µL glycogen (Roche). The resulting purified DNA was redissolved in 5 –

10 µL of H_2O or TE as indicated. Purity and concentration of the product were

tested using polyacrylamide gel electrophoresis as described above.

2.3.7 DNA sequencing

Manual DNA sequencing was used to determine orientation of cloned

DNA during recombinant plasmid construction. Sequencing reactions were

performed in 0.6 µL tubes (Rose) or 0.2 µL tubes as indicated in either the

Techne PHC-2 Thermocycler or the MJ Research Minicycler™. All sequencing

reactions were performed using the Thermo Sequenase Radiolabeled Terminator

Cycle Sequencing kit (USB) and were carried out as per manufacturer's

instructions. Typical reaction mixtures contained 20 mM Tris-HCl (pH 9.5), 6 mM

$MgCl_2$, 2 pmol of oligonucleotide primer, 2 µM dNTPs, 0.02 µM ddNTPs, 8 U

Thermo Sequenase™ and between 50 and 500 ng of template DNA. The

reactions were subjected to a 2 minute denaturation at 95°C, followed by 30

cycles of denaturation for 30 seconds at 95°C, primer annealing for 30 seconds

at 52°C and elongation for 1 minute at 72°C. Upon completion, 4 µL of Stop

Solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05%

xylene cyanol FF; USB) were added to each reaction. Between 3 – 5 µL of each

reaction were electrophoresed on a formamide sequencing gel (19:1, acrylamide: N, N'-bisacrylamide, 8.3 M urea, 1 X TBE) at 35 watts until the bromophenol blue dye had reached the bottom of the gel. The gel was removed from the electrophoresis apparatus and placed on a sheet of 3MM Whatman No.1 filter paper and covered with Saran Wrap. The gel was dried on a Bio-Rad gel dryer with Savant pump under vacuum at 80˚C. To visualize the sequence, the dried gel was placed on a phosphorscreen overnight and scanned by a Molecular Dynamics Model 445 SI phosphorimager. Analysis of data was done using Imagequant™ software.

2.3.8 Colony DNA transfers

For screening large numbers of *E. coli* colonies for cloned DNA, colony DNA hybridization analysis was used. Selected *E. coli* colonies were streaked with a sterile toothpick onto Hybond-N (Amersham) nylon membranes (containing numbered grids) which had been placed onto LB plates containing the appropriate selective antibiotic and were duplicated onto numbered agar plates containing the identical media composition. Once colonies had grown, the membranes were removed and placed, colony side up, onto Whatman paper saturated with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes. Following the denaturation step, the membranes were transferred to Whatman paper soaked in neutralizing solution (3 M NaCl, 0.5 M Tris) for 5 minutes. This step was repeated by transferring the membranes to a second sheet of neutralized Whatman paper for an additional 5 minutes. Finally the neutralized

membranes were placed in a 2 X SSC solution (0.3 M NaCl, 0.03 trisodium citrate) and colony debris was scrubbed, with a gloved hand, from the membranes so as not to interfere with radioactive probing of the DNA. Excess moisture was removed from the membranes with paper towels and DNA was cross-linked to the nylon membrane at 150 mJoules using a Bio-Rad GS Gene Linker. The membranes were wrapped in Saran Wrap and stored at –20˚C.

2.3.9 Southern hybridization analysis

Southern hybridization analysis was performed as described in Hopwood *et al.* (Hopwood *et al.*, 1985) as a modification of the Southern procedure (Southern, 1975). DNA was separated by electrophoresis on a 1% TBE agarose gel at 36 volts until the dye had just reached the bottom of the gel. This gel was stained as described in section 2.3.4, and photographed to determine the migration of the DNA and molecular weight marker (MWM III, Roche). Once photographed, the gel was soaked, with gentle rocking, for 2 x 10 minutes in 0.25 M HCl to aid in DNA transfer. This was followed by treatment with denaturing solution (see section 2.3.9) for 2 x 15 minutes, also with gentle rocking, rinsing with distilled H_2O in triplicate and finally treating with neutralizing solution (section 2.3.9) for 20 minutes. The transfer apparatus consisted of 2 pieces of 3 MM Whatman No.1 filter paper, saturated with 20 X SSC, wrapped around a glass plate with the ends dipped into a solution of 20 X SSC to form a wick. The treated gel was placed well-side down onto the filter paper to facilitate transfer of the DNA to a nylon membrane (Hybond™-N; Amersham), which was trimmed to

be the same size as the gel. This membrane was placed directly onto the gel.

To help facilitate the transfer, two pieces of Whatman paper (same approximate

size as the membrane) were placed on top of the membrane, followed by paper

towels (10 – 15 cm high) and a glass plate which supported a suitable weight.

Following transfer of at least 6 hours, the apparatus was disassembled, the gel

lanes marked onto the membrane and the DNA was cross-linked to the

membrane as described in section 2.3.9. The damp membranes were wrapped

in Saran Wrap and stored at –20˚C.

2.3.10 Preparation of labeled DNA probes

Random primer labeling was used to label DNA probes used to detect

DNA of interest in colony hybridizations and Southern hybridizations, as well as

to detect RNA transcripts by northern hybridization. This procedure for internally

labeling the DNA fragment is as described in Feinberg and Vogelstein (1983;

modified by Roche). Nine microlitres of DNA probe is heated for 10 minutes at

90˚C for denaturation and transferred immediately to ice. To this mixture, 2 µL of

hexanucleotide mix (Roche), 3 µL of 0.125 mM dNTP's (dATP, dGTP, dTTP), 5

µL (50µCi) of $[\alpha^{32}P]$-dCTP (Amersham) and 1 µL (2 U) Klenow (Roche) were

added. The entire reaction mixture was incubated for 3 hours at 37˚C or

overnight at room temperature. The unincorporated nucleotide was separated

from the labeled DNA using a Micro Biospin® 6 chromatography column (Bio-

Rad). Following the separation, 1 µL of purified DNA probe was Cerenkov

counted in a Beckman LS 3801 scintillation counter.

Oligonucleotide probes for use in RT-PCR, S1 nuclease mapping, and primer extension analysis were 5' end-labeled (Chaconas and van de Sande, 1980; modified by Roche). Final reactions typically contained 2 μL of DNA probe (10 – 50 pmol), 50 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT; pH 7.5, 5 μL (50 μCi) [γ ^{32}P]-dATP (ICN) and 2 U polynucleotide kinase (Roche). The kinase enzyme was added in two aliquots, one unit per addition. The first unit of enzyme was added upon mixing all other reaction components and this mixture was incubated at 37°C for 15 minutes. At this point, the second unit of kinase enzyme was added to the mixture and the reaction was incubated for an additional 15 minutes at 37°C. Following this incubation, the reaction volume was made up to 50 μL with sterile milli-Q water and the probe was precipitated on ice using 1/10 volume 3M sodium acetate, 2 volumes 95% ethanol and 20 μg glycogen (molecular biology grade, Roche). The entire DNA pellet was Cerenkov counted as described in section 2.3.11 and the DNA was dissolved in milli-Q water to either 50 000 cpm/ 2μL or 10 pmol DNA / μL as indicated.

2.3.11 DNA hybridizations

Colony hybridizations were performed in deep glass petri-dishes using 10-20 mL hybridization buffer [3 X SSC (0.45 M NaCl, 0.045 M trisodium citrate), 4 X Denhardt's solution {0.08 % polyvinylpyrrolidone (MW 360000)}, 100 μL/10 mL denatured salmon sperm DNA and deionized formamide when necessary]. Prehybridization was carried out for at least 3 hours in a shaking water bath (Hot Shaker; Bellco Biotechnology), before addition of the labeled DNA probe.

All northern and most Southern transfer hybridizations were carried out in glass hybridization tubes in the same hybridization buffer described above. Prehybridization of the membranes occurred in a hybridization incubator (Robbins Scientific) for not less than 3 hours. When necessary, Southern transfer hybridizations were conducted in small glass Pyrex® dishes using 30 mL of hybridization solution. Prehybridization took place in the shaking water bath as with colony hybridizations.

Hybridization of all transfers using labeled DNA fragments as probes was performed at approximately 25°C below the calculated melting temperature of the probe. The specific melting temperature for each probe was calculated using the following formula: $Tm = 81.5\ °C + 16.6\log M + 0.41(\%G+C) - 500/n - 0.61(\%$ formamide), where M is the ionic strength (0.45 for 3X SSC) and n is the shortest region of homology (Hopwood $et\ al.$, 1985). After at least 8 hours of hybridization, the membrane was washed with 2 X SSC, 0.1% SDS for 1-2 X 30 minutes, followed by 1-2 X 15 minutes with 0.2 X SSC, 0.1% SDS. Washed membranes were wrapped in Saran Wrap and exposed to a phosphorscreen. Visualization and analysis were carried out as in section 2.3.8.

When necessary, radioactive DNA was stripped from the membrane by soaking in 0.1% SDS that was brought to boiling.

2.4 RNA Analysis

2.4.1 Isolation of RNA

Streptomyces RNA was isolated using a modified version of the Kirby *et al.* (1967) procedure, detailed in Hopwood *et al.* (1985), with a few changes. Cultures were grown on cellophane discs placed on R2YE agar plates. At specific harvesting times, cultures were scraped from the cellophane using a spatula, which had been baked at 200°C for at least 2 hours. The culture was placed into a 20 mL universal vial containing 5 mL Kirby's mix (1% sodium triisopropylnaphthalene sulfonate, 6% sodium – 4 – amino salicylate, 6% neutral phenol, buffered in 50 mM Tris-HCl, pH 8.3) and glass beads to a height of 1 – 2 cm from the bottom of the vial. The cell suspensions were vortexed for 4 X 30 second intervals with all vials kept on ice in the interim. Five milliliters of phenol chloroform were added to the vials and all mixtures were vortexed for 2 additional 30 second intervals. The homogenates were transferred, using 5 mL pipettes (baked as above), to polypropylene tubes and were centrifuged at 4°C for 5 – 10 minutes at 8500 rpm in a Beckman J2-H5 centrifuge with JA20 rotor. The resulting aqueous layers were transferred to new polypropylene tubes containing 5 mL phenol chloroform, vortexed for 30 seconds and centrifuged as above. This process was repeated until no interface remained between aqueous and solvent layers. The nucleic acids present in the resulting aqueous solution were precipitated using 1/10 volume sodium acetate and 1 volume isopropyl alcohol. The precipitation was carried out at –70°C overnight after which the solutions were thawed and centrifuged at 4°C for 10 minutes at 8000 rpm (see above).

The resulting pellets were rinsed with 95% ethanol and dried until no odor of alcohol remained. The pellets were then redissolved in 450 μL DEPC-treated H_2O and transferred to RNAse-free 1.6 mL Eppendorf tubes. To remove DNA contamination from the nucleic acid solution, 1/10 volume of 10X DNAse Buffer (0.5 M Tris-HCl, pH 7.8, 0.05 M $MgCl_2$), and 7 units of RNAse-free DNAse (Roche) were added. This reaction was incubated at room temperature for 30 minutes at which time an additional 7 units of DNAse were added and the reaction incubated an additional 30 minutes at room temperature. Upon completion of the DNA digestion, the reaction was extracted twice with phenol chloroform to remove the enzyme and extracted 3 times with chloroform to remove contaminating phenol from the RNA. The RNA was precipitated on ice for 20 minutes using 1/10 volume sodium acetate and 1 volume of isopropyl alcohol. The precipitate was collected by centrifugation, rinsed with DEPC-treated 80% ethanol, dried and redissolved in 100 – 200 μL DEPC-treated H_2O. The RNA was quantified by spectrophotometry and qualitatively examined by agarose gel electrophoresis. Prior to storage at –80°C, 1/10 volume sodium acetate and 1 volume of isopropyl alcohol were added.

2.4.2 Labeling of RNA riboprobes

The riboprobe northern hybridization method for detecting RNA required the synthesis of RNA probes using the Riboprobe® System – T7 labeling kit (Promega). Reaction mixtures consisted of 0.2 – 1.0 μg linearized template DNA, 1X Transcription Optimized 5X Buffer (Promega), 10 mM DTT, 20 units

Recombinant RNasin Ribonuclease Inhibitor, 0.5 mM NTP mix (ATP, GTP, CTP),

11.5 μM UTP, (50 μCi) $[\alpha^{32}P]$ – UTP and 20 units T7 RNA polymerase

(Promega). The entire mixture was incubated for 1 hour at 37˚C. Labeled RNA

was precipitated using DEPC-treated sodium acetate, isopropyl alcohol and

RNAse-free glycogen to separate it from unincorporated nucleotide. The RNA

probe was redissolved in 20 μL DEPC-treated H_2O. To determine the efficiency

of labeling, 2 μL of probe were Cerenkov counted as above.

2.4.3 Northern hybridization analysis

To detect the presence of a specific RNA transcript among total

Streptomyces RNA (isolated in section 2.4.1), northern hybridization analysis, as

described by Williams and Mason (1985), was performed. Thirty micrograms of

each RNA sample were dissolved in 2.5 μL of DEPC-treated milli-Q water. To

denature the RNA, the samples were heated for 1 hour in a solution containing 1

M glyoxal, 50% DMSO and 10 mM $NaPO_4$ (pH 7.0) (6 mM Na_2HPO_4, 4 mM

NaH_2PO_4). Molecular weight markers III and V (625 ng, Roche) served as size

markers and were carried through the same treatments as the RNA samples. All

glyoxalated samples (RNA and DNA) were separated for analysis by

electrophoresis on a 1.25% agarose gel using a 10 mM $NaPO_4$ (pH 7.0)

recirculating buffer system. Electrophoresis occurred at 58 volts until the blue

dye had reached 2/3 distance of the gel. RNA was transferred overnight to a

Hybond-N (Amersham) membrane using the same transfer apparatus described

for Southern transfers in section 2.3.10. The RNA was UV cross-linked to the

membrane and glyoxal was removed by baking the membrane at 80°C for 1 hour under vacuum.

2.4.4 Northern hybridization analysis using riboprobes

When screening RNA samples with an RNA riboprobe (section 2.4.2), an alternative method of northern hybridization analysis was used (Ausubel *et al.*, 1995). Thirty microgram samples of RNA were first dissolved in 5 µL of DEPC-treated milli-Q water and then heated to 65°C for 15 minutes in a solution containing 50% formamide, 16% formaldehyde, 7% glycerol, 0.2% bromophenol blue and 1 X MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). The samples were electrophoresed on a 1.2% agarose gel containing 5% formaldehyde in a 1 X MOPS buffer system at 58 volts as in section 2.4.3. The RNA was then transferred to a Hybond-N membrane (Amersham), UV cross-linked and baked. Pre-hybridization at 65°C in buffer containing 50% formamide, 5 X Denhardt's, solution, 0.2% SDS, 2 X SSC, 100 µL/ 10mL denatured salmon sperm DNA, for at least 3 hours before adding the labeled RNA riboprobe. Hybridization was carried out overnight at 65°C after which, the membrane was washed using 0.1 X SSC and 0.1% SDS for at least 2 X 20 minutes. The membrane was wrapped in Saran Wrap and exposed to a phosphorscreen overnight. Visualization and analysis are as described in section 2.3.8.

2.4.5 S1 nuclease protection assays

Low resolution S1 nuclease protection of RNA 5' ends was used in an attempt to visualize expression levels of the helicase SCH5.13 throughout the life cycle of *Streptomyces coelicolor*. Fifty microgram samples of RNA were dissolved in 2 µL of end-labeled DNA probe solution (50 000 cpm in DEPC-treated milli-Q water). The nucleic acid mixture was dried using a Speed Vac Concentrator (Savant) and redissolved in 20 µL of S1 hybridization buffer (3.2 mM PIPES buffer (pH 6.4), 0.4 M NaCl, 1 mM EDTA, 80% formamide). The S1 hybridization mixture was heated to 80°C in a water bath for 20 minutes at which time the sample was mixed by vortex and microcentrifuged before being placed at 80°C for an additional 10 minutes. Following this, the temperature of the water bath was reduced to 5°C above the calculated melting temperature of the DNA probe (for calculation see section 2.3.12; M is 0.4 for S1 hybridization buffer) where it remained for the duration of the night. The following morning the tubes were then placed on ice and 200 U of S1 nuclease (Sigma) in digestion buffer (0.28 M NaCl, 30 mM sodium acetate (pH 4.4), 4.5 mM $(CH_3CO_2)_2Zn$, 20 µg partially-cleaved denatured calf thymus DNA) were added. The digestion reaction was incubated at 37°C for 45 minutes at which time the reaction was terminated by the addition (on ice) of 2.5 M ammonium acetate and 0.05 M EDTA. The reaction was then subjected to phenol chloroform extraction to remove enzymes, followed by chloroform extraction to remove contaminating phenol. The DNA/RNA duplex was precipitated for 30 minutes at −20°C using 1/10 volume of sodium acetate, 1 volume of isopropanol and 1 µL of glycogen

(Roche). Precipitated duplex was collected by centrifugation and rinsed with DEPC-treated 80% ethanol prior to being dissolved in 3 µL loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue). This mixture was heated to 90°C for 5 minutes and then placed directly on ice before being loaded onto a 6% acrylamide sequencing gel and electrophoresed at constant power (35 watts) until the bromophenol blue had just reached the bottom of the gel. Once electrophoresis was complete, the gel was placed onto 3MM Whatman No.1 filter paper, dried and placed on a phosphorscreen for visualization.

2.4.6 Primer extension analysis

Primer extension analysis was done to determine the expression pattern of helicase SCH5.13. The procedure described by Gabriela Kelemen (personal communication) was used in most cases. Forty micrograms of total RNA was dissolved in a total volume of 20 µL containing 1-5 pmol of end-labeled oligonucleotide primer, 1 X SB buffer (60 mM NH_4Cl, 10 mM Tris-acetate (pH 7.4), 6 mM 2-mercaptoethanol; Hartz *et al.*, 1988), and 16 units of RNA Guard (Amersham). The mixture was incubated at 90°C for 5 minutes to denature the probe, then transferred to a 75°C water bath that was allowed to cool to 55°C to allow for the DNA probe to anneal to the RNA transcript. The samples were centrifuged and put on ice. A 5 µL aliquot of the annealed reaction mixture was added to 10 µL of Reverse Transcriptase (RT) Assay solution (1 X SB buffer, 15 mM Mg-acetate, 0.75 mM dNTP's (dATP, dCTP, dGTP, dTTP), 16 units of RNA

Guard (Amersham), 12.5 units of AMV reverse transcriptase (Roche). The RT

reaction was incubated at 45°C for 30 minutes. Following this incubation, 6 µL of

loading dye (section 2.4.5) was added to each reaction. The tubes were then

placed, with caps open, at 80°C for 20 minutes to concentrate the mixture. Two

microlitres of the concentrated product was loaded onto a 6% acrylamide

sequencing gel and electrophoresed as in section 2.3.8. Following

electrophoresis, the gel was dried onto Whatman filter paper and exposed to X-

ray film (Kodak X-OMAT AT film) at room temperature and developed in a FUGI

RGII X-ray film processor.

Alternatively, the procedure described by Penfold *et al.* (1996) was used,

also with 40 µg of RNA and the resulting gel was placed on a phosphorscreen for

visualization and analysis as described previously.

2.4.7 Reverse-transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was used to visualize the transcript expression pattern of

helicase SCH5.13 throughout the *Streptomyces* developmental cycle as well as

to narrow down the location of the transcription start site of SCH5.13. A primer,

internal to the helicase transcript, was designed such that it could be used to

generate a cDNA of the 5' end. Using this primer, reverse transcription of the

helicase transcript was set up using the *C. therm* Two-Step RT-PCR kit from

Roche as per manufacturer's specifications. Three micrograms of total RNA

isolated in section 2.4.2 was used as template for the reaction that was incubated

at 69°C for 30 minutes. Five microlitres of these RT reactions were used in the

PCR amplification step described in detail in section 2.3.3. Following amplification of the cDNA, 5 μL of loading dye (0.25% bromophenol blue, 40% sucrose) was added to the samples before loading 15 μL onto a 5% polyacrylamide gel. The gel was electrophoresed for 2 hours at 200 volts after which it was dried onto Whatman filter paper and placed on a phosphorscreen overnight. Visualization and analysis are as described earlier (section 2.3.8).

2.5 Protein Isolation and Enzyme Assays

2.5.1 Cell free extract preparation from Streptomyces

Cultures grown on cellophane discs placed onto R2YE agar plates were harvested by scraping with a sterile spatula into 1.6 mL Eppendorf tubes. Lysing buffer (0.1 M HEPES pH 7.2, 1 mM Pefabloc, 1 mM PMSF, 1 μM Pepstatin A, 0.5 mg/mL Lysozyme) was added to the culture (1 mL maximum) and incubated for 10 minutes at 37˚C. The tubes were removed from the water bath and placed on ice before sonicating using a Branson Sonifier 450 with a micro tip at level 1 for up to 5 X 15-second intervals. The tubes were kept on ice during sonication and in the interim. Once sonication was complete, the samples were centrifuged for 10 minutes at 4˚C to sediment the cell wall material. One hundred microlitre aliquots of the protein supernatants were transferred to 1.6 μL Eppendorf tubes and stored at -86˚C.

2.5.2 Quantification of total protein in cell free extracts

Quantification of total protein isolated in section 2.5.1 was performed using the dye-binding assay (Bradford, 1976) with the Bio-Rad Protein Assay kit as per manufacturer's specifications. Bovine Gamma Globulin (Bio-Rad) was used to construct a standard curve of protein verses absorbance at 595 nm. The standard curve allowed the protein content in µg/µL to be calculated as follows: µg protein/N x Y/1000, where N is the volume of diluted protein assayed and Y is the dilution factor used in N.

2.5.3 Catechol dioxygenase activity assays

Quantitative expression from both the helicase and *bldG* promoters in *Streptomyces* was measured using the procedure described by Zukowski *et al.* (1983). One hundred microlitres of cell free extract was added to a mixture composed of: 2.9 mL of KPO$_4$ (pH 6.8) (40 mM K$_2$HPO$_4$, pH 8.8, 60 mM KH$_2$PO$_4$, pH 4.4), 10 µL 20 mM catechol (in 95% ethanol) for a final solution volume of 3.0 mL. The reaction was mixed and placed in a spectrophotometer to measure the change in absorbance at 375 nm over 8 minutes. The slope was calculated as A$_{375}$ per minute. Under the above conditions, the molar extinction coefficient (ε) of 2-hydroxymuconic semialdehyde is reported as 3.3 x 10^4 M^{-1}cm^{-1} (Sala-Trepat and Evans, 1971). Activity of the enzyme was calculated in mU/mg (milli-units per mg of total protein where one milli-unit is defined as the formation of 1 nmol of 2-hydroxymuconic semialdehyde in one minute) using the formula: [(Slope x D

x 3) $10^6 / \varepsilon$]/ P, where D is the dilution factor of the protein solution and P is the protein concentration in mg/mL (calculated in section 2.5.2).

Chapter 3:

Results

3. RESULTS

During the initial cloning and sequencing of the *bldG* locus, a partial open reading frame (ORF) was identified that was transcribed divergently from the *bldG* gene. The entire ORF DNA sequence was retrieved from the *S. coelicolor* genome sequencing project database (http://www.sanger.ac.uk/ Projects/S_coelicolor/) and the deduced amino acid sequence was used to search the databases using Blast. The results revealed that the ORF, designated SCH5.13, showed high sequence similarity to a number of known and putative proteins that unwind RNA duplexes, often through the use of ATP or another nucleotide triphosphate. Although RNA helicases have been implicated in cellular processes such as translation initiation, mRNA turnover and development in all branches of life, there have been no such proteins described in *Streptomyces*. FRAME analysis of the *bldG* locus revealed a close proximity of the SCH5.13 and *bldG* ORFs with the *Streptomyces* genome sequencing project identifying a GTG codon for translation initiation of the SCH5.13-encoded protein (Fig 3.1). However, the FRAME plot shown in Fig. 3.1 also revealed a downstream in-frame ATG codon with the potential to be a putative start codon. Either of these potential translation start codons are close enough to the *bldG* translation start codon that the promoters of these two genes could overlap, suggesting the possibility of coordinate control of transcription of these two genes. Because *bldG* is a known regulator of *S. coelicolor* morphological and physiological differentiation, it is attractive to suggest that the putative RNA helicase encoded by the SCH5.13 ORF would also play a part in regulation of the

Figure 3.1: FRAME analysis of the *bldG* locus. ATG and GTG codons present in each frame are represented above the main plot. The GTG codon suggested to initiate translation of the SCH5.13 ORF is marked with a black arrow while the downstream ATG codon that has since been implicated as a potential start codon is marked by a black arrow with an asterisk. The extent and orientation of the putative protein – encoding ORFs are indicated by the large coloured arrows.
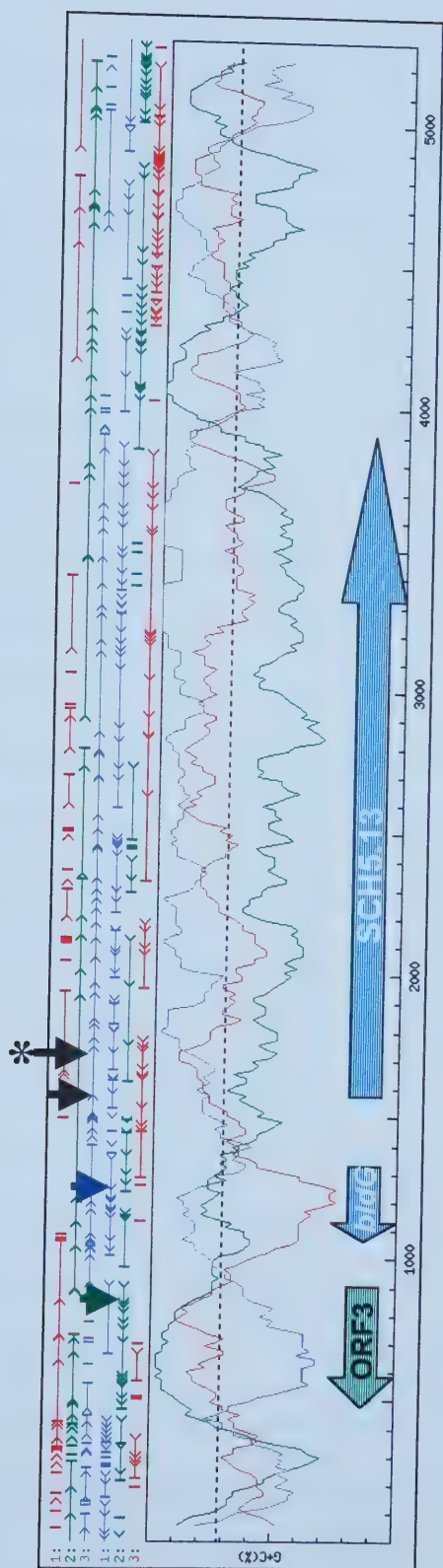
cell cycle of this complex prokaryote. Therefore, the purpose of this thesis project was to determine whether this putative RNA unwinding enzyme plays a role in *S. coelicolor* development through transcriptional analysis and isolation of SCH5.13 null mutants.

3.1. Growth Phase Dependence of SCH5.13 Transcription in S. coelicolor

3.1.1. Northern hybridization analysis

To determine whether the putative RNA helicase encoded by the SCH5.13 gene is involved in differentiation, experiments were designed to see if the gene was expressed temporally and if so, when during the *S. coelicolor* life cycle it was transcribed. A common method for visualizing the transcription pattern of a gene is to use northern hybridization analysis where total RNA from various times post-inoculation is probed with a labeled DNA fragment from the gene of interest. In this case, in order to generate the probe, a 225 bp DNA fragment corresponding to the 5' end of the putative helicase coding sequence was amplified using primers BKL87 and BKL88 (Table 2.5). To avoid contamination, and thus interference, from any non-specific PCR amplification products, this DNA was purified from a polyacrylamide gel by crushing and soaking (Section 2.3.6). Finally, a radioactive probe, specific for the putative helicase gene, was generated by random primer labeling a 1 µL aliquot of the purified DNA.

Total RNA was isolated from *S. coelicolor* J1501 at various times post-inoculation over a 48 hour time course. Thirty micrograms of RNA from each

sample time were prepared for northern analysis as described in Section 2.4.3

and electrophoresed along with DNA markers that had been subjected to the

same glyoxal denaturation. After transfer of the nucleic acids to a nylon

membrane, hybridization with the labeled 225 bp DNA probe was carried out at

45°C in 50% formamide hybridization buffer.

Unfortunately, no hybridizing transcript was detected and so the

membrane was stripped and probed at lower stringency using 30% formamide

hybridization buffer. Again, no transcripts corresponding to the SCH5.13 ORF

were detected. Further attempts to detect the transcript using 40 μg amounts of

sample RNA from a separate time course also failed.

3.1.2. S1 nuclease protection assays

It was possible that the inability to detect SCH5.13-encoded transcripts

was due to degradation of the long mRNA transcripts (the expected transcript

length for the SCH5.13 ORF is at least 2400 nt) within the total RNA samples.

Because of this, S1 nuclease protection assays were attempted since this

method requires only that the 5' end of the transcript remain intact in order to be

detected. A 547 bp DNA probe was generated by PCR amplification using

oligonucleotide primers BKL80 and BKL87 (Table 2.5). The resulting DNA

extended 323 bp upstream from the putative ATG helicase translation start

codon and 92 bp into the divergently transcribed *bldG* coding sequence. This

probe design was chosen to permit detection of the putative helicase transcript

even if degradation of the 3' end had occurred since it required that only 200

nucleotides at the 5' end of the greater than 2400 nucleotide transcript remain intact.

The 547 bp DNA probe was end-labeled and hybridized overnight at 55°C to 50 μg aliquots of total RNA samples harvested at several times post-inoculation. After the nucleic acid samples were treated with S1 nuclease as described in Section 2.4.5, they were electrophoresed on a sequencing gel alongside end-labeled DNA markers. As with the northern analysis, SCH5.13 transcripts could not be detected even upon repeating the procedure several times with independently isolated RNA samples.

### 3.1.3. Northern analysis using RNA probes

One possible reason for the failure to detect the SCH5.13 transcript using either northern or S1 nuclease protection analysis, was that the helicase mRNA was present as a very small fraction of the total RNA preparation, and that the hybridization between the DNA probe and the helicase transcript was not strong enough to allow detection. To test this theory, a radioactive riboprobe was synthesized using the strategy outlined in Fig. 3.2.

Using the oligonucleotide primers BKL82 and BKL91, a 750 bp region, that included 150 nucleotides of DNA upstream of the putative helicase gene start codon, was amplified by PCR. *Xba*I and *Hind*III restriction sites were engineered into the primers and allowed for directional cloning of the amplified fragment into the vector pBluescript KS. This parent vector is unique in that both the T3 RNA polymerase promoter and the T7 RNA polymerase promoter flank its

Figure 3.2: Schematic drawing of the overall strategy used to construct a radioactively labeled riboprobe. (A) PCR using oligonucleotide primers BKL82 (*Xba*I site engineered into its sequence) and BKL91 (*Hind*III site engineered into its sequence) was used to amplify a 750 bp segment of DNA. Oligonucleotide primers are represented by small black arrows which indicate direction of synthesis and approximate region of homology with the template DNA. The amplified region included 150 bp of the putative helicase – *bldG* intergenic region (shown as ▨▨▨) and 600 bp of helicase gene sequence (shown as ▢). Digestion of the amplified DNA with *Hind*III and *Xba*I allowed easy ligation of the fragment into the corresponding sites in the pBluescript KS polylinker (not all restriction sites shown) which is flanked by T3 and T7 RNA polymerase promoters (shown as large black arrows that indicate the direction of RNA transcription). (B) The recombinant pBluescript KS plasmid is designated pAU220. Black triangles represent the RNA polymerase promoters flanking the inserted DNA. The inserted DNA includes a unique *Sph*I restriction site that includes, within its recognition sequence, the proposed ATG start codon for the putative helicase gene. (C) pAU220 linearized with *Sph*I. Linearization allowed the synthesis from the T7 RNA polymerase promoter of RNA transcripts complementary to the putative helicase gene sequence. Radiolabeled UTP (represented as *) promotes specific, uniform labeling of the riboprobe.
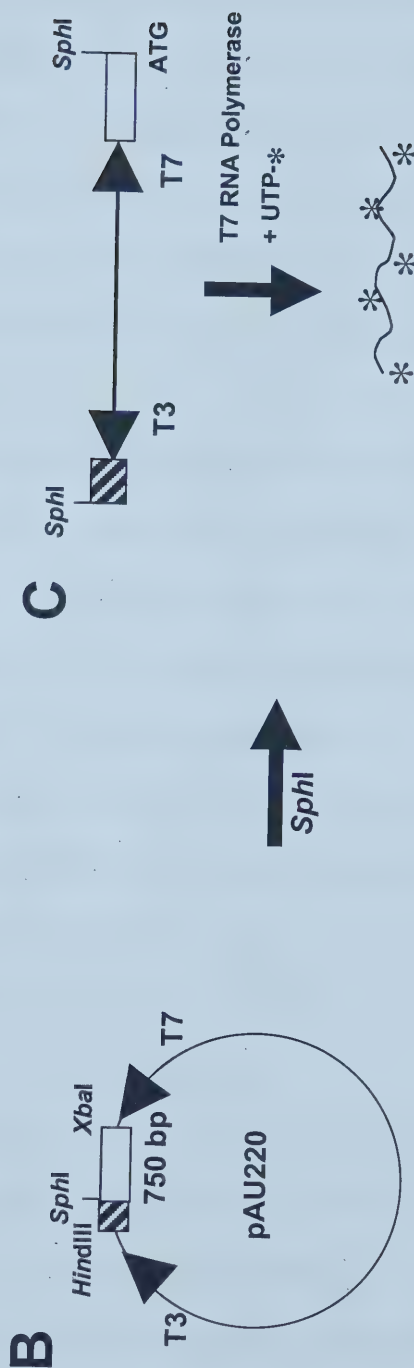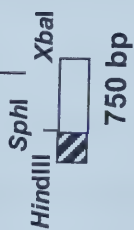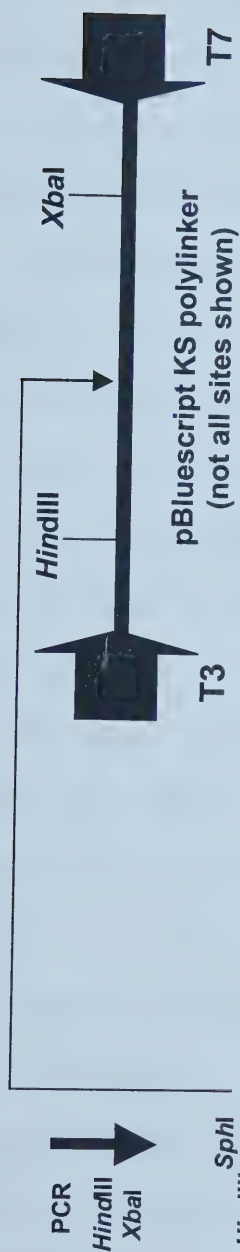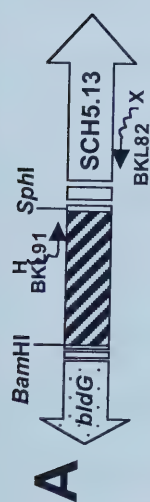
multiple cloning site. Conveniently, the proposed ATG start codon of the putative

helicase gene was contained within a unique *Sph*I restriction site, which allowed

for linearization of the resulting vector (pAU220). This also ensured that the RNA

probe transcribed from the T7 RNA polymerase promoter consisted of only

SCH5.13 sequence so that no false positive results could result. Including ^{32}P-

UTP in the labeling reaction allowed for strong, uniform labeling of the RNA

probe which was complementary to 600 nucleotides at the 5' end of the putative

helicase gene.

The labeled riboprobe was used in northern hybridization experiments to

probe 20, 30 and 35 µg aliquots of RNA from the same time course samples

used for northern and S1 nuclease protection analysis, described above, as well

as two independently performed RNA timecourses. In addition to the standard

northern blotting procedure described above, an alternative method was

attempted which uses a formaldehyde-containing buffer to heat RNA samples

and a formaldehyde-containing agarose gel electrophoresis step (Section 2.4.4).

As with the other procedures, no signal could be detected.

3.1.4. Promoter probe analysis

3.1.4.1. Single copy expression using the integrative vector pSET152

Since detection of the SCH5.13 transcript had proved to be problematic

using various RNA analysis techniques a new approach was needed to

determine whether this gene is temporally expressed during the *S. coelicolor* life

cycle. Promoter probe analysis was the method of choice and involved cloning

the promoter of SCH5.13 into a vector such that activity from this promoter would direct the transcription and thus expression of the *xylE* reporter gene. *xylE* encodes catechol 2,3 dioxygenase, an enzyme which converts the colourless substrate catechol to the yellow compound 2-hydroxymuconic semialdehyde which can be quantified spectophotometrically. To avoid problems with plasmid copy number variability at different stages of colony growth (Kieser *et al.*, 1982), plasmid pSET152 was used as a parent vector for single copy promoter probe construction. This vector will integrate into the *S. coelicolor* chromosome at the φC31 phage attachment site and thus will be replicated only as often as the chromosome itself divides. In addition, the vector contains the gene for apramycin resistance, Ap^R, which can be selected in *Streptomyces*.

The intergenic region between the putative helicase and *bldG* start codons was amplified using primers JWA19 and JST1 and was subsequently digested with *Bam*HI to result in a DNA product of 240 bp in length (Fig. 3.3A). This fragment was blunt-ended and ligated into pSET152 which had been digested with *Eco*RI and also blunt-ended (Fig. 3.3B). Plasmid DNA was isolated from white, apramycin resistant colonies and digested first with *Sph*I (Fig. 3.4B) to determine if the promoter DNA was present in the pSET152 polylinker. Since both the pSET152 vector and the inserted intergenic region contain *Sph*I sites, digestion with *Sph*I generates two bands on the gel (Fig. 3.4B; lanes 3,4 and 8). Plasmid DNA from the three colonies containing the intergenic region cloned into pSET152 was then digested with both *Sph*I and *Xba*I (Fig. 3.4C) to confirm the orientation of the inserted DNA. No fragment seen (as in lane 3 of Fig. 3.4C)

Figure 3.3: Strategy used to construct the vectors pAU230 and pAU231. (A) Primers JWA19 and JST1 (*Bam*HI site engineered into the 5' end sequence) were used to amplify the entire helicase – *bldG* intergenic region. This generated a 350 bp fragment of DNA that was subsequently digested with *Bam*HI to leave a 240 bp fragment. Primers are represented by small black arrows, which indicate direction of synthesis and approximate region of homology with the template DNA. Finally, this intergenic region DNA was blunt-ended to ready the fragment for ligation into pSET152. (B) Restriction map of parent vector pSET152 (Bierman *et al.*, 1992). The plasmid was digested with *Eco*RI and then blunt-ended to accommodate the intergenic region DNA fragment from (A). (C) pSET152 polylinker. The blunt intergenic region DNA could ligate in two orientations with respect to the putative helicase promoter. The recombinant vector containing this DNA with the helicase promoter directed toward the *Bam*HI site, was renamed pAU230. The vector containing the inserted DNA in the opposite, orientation with the *bldG* promoter directed towards the *Bam*HI site was renamed pAU231.
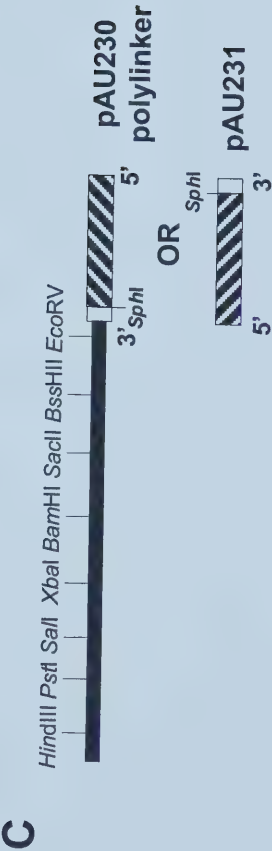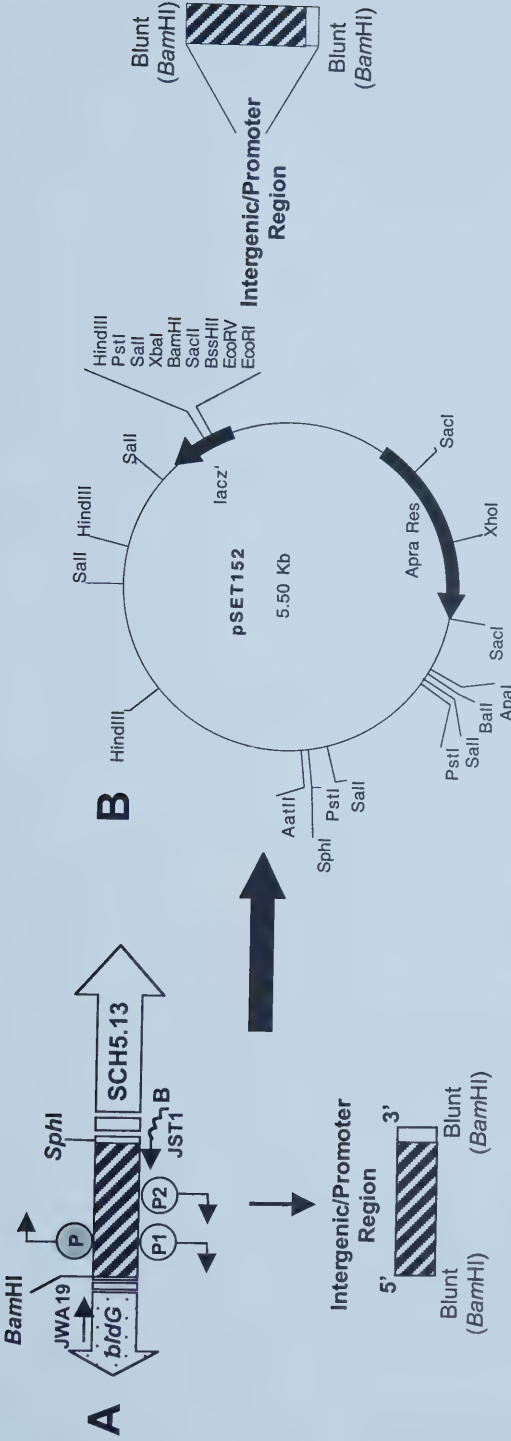
Figure 3.4: Verification of intergenic region DNA inserted into pSET152. (A) Schematic drawing of recombinant vectors pAU230 and pAU231 and the approximate location of relevant restriction sites used for confirmation of construction. (B) 0.7% TBE agarose gel showing SCH5.13 – *bldG* intergenic region DNA has been ligated into pSET152. Plasmid DNA was digested with *Sph*I to determine if the intergenic region DNA had been inserted into the pSET152 polylinker. Double-dashed arrows (lanes 4 and 8) indicate DNA bands resulting from pAU231 digestion the while dotted arrow (lane 3) denotes the DNA bands resulting from pAU230 digestion. λDNA digested with *Pst*I (lane M) was used for molecular weight determination. Parent vector pSET152 digested with *Sph*I (lane C) served as a negative control. (C) 5% polyacrylamide gel confirming the presence and orientation of the SCH5.13 – *bldG* intergenic region DNA in pSET152. Plasmid DNA from samples 3, 4, and 8 in (B) was digested with *Xba*I and *Sph*I. Molecular weight marker V (Roche) was used for size determination of the released bands. The double-lined arrow (lanes 4 and 8) indicates release of a band of ~240 bp, confirming pAU231 construction. The inability to resolve a fragment in lane 3 was taken as confirmation for pAU230 construction given the proximity of the restriction sites for *Sph*I and *Xba*I (~20 bp) if the fragment had the helicase promoter directed toward the *Bam*HI site. Lane C contains pSET152 digested with *Xba*I and *Sph*I as a negative control.
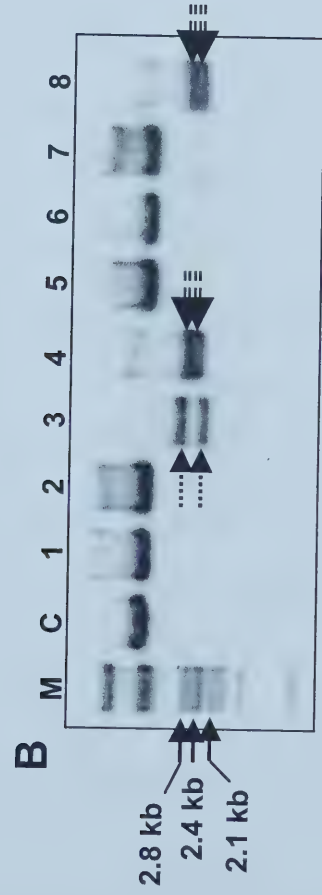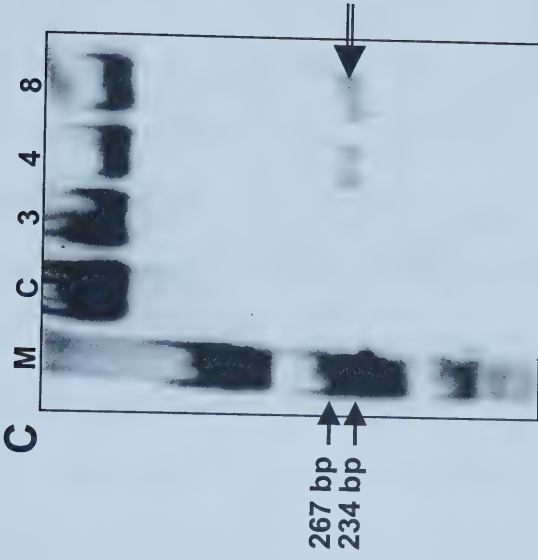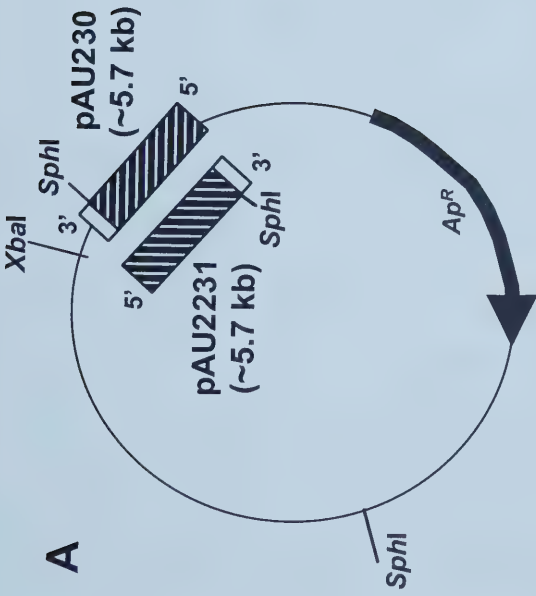
was taken as confirmation of the intergenic region ligated into pSET152 in the orientation with the helicase promoter directed towards the *Bam*HI site (pAU330; Fig. 3.3C), because the resulting DNA fragment would be only about 20 bp, which is too small to resolve on the polyacrylamide gel. Of the three isolated plasmid preparations, one that showed the proper DNA banding pattern was confirmed to contain the helicase promoter and was named pAU230. The other two clones harboured the DNA in the opposite orientation that is, in the orientation with *bldG* promoter directed towards the *Bam*HI site (diagrammed in Fig. 3.3C) in the polylinker was renamed pAU231.

For construction of the helicase promoter probe plasmid, pAU221, the *xylE* gene was isolated from vector pIJ4083 (Clayton and Bibb, 1990) using *Bam*HI and *Bgl*II and was subsequently ligated into the *Bam*HI restriction site in the polylinker of pAU230 (Fig.3.5A). Transformation into *E. coli* DH5α yielded numerous apramycin resistant colonies that were screened, by spraying with 0.5 M catechol, for insertion of *xylE*. Those colonies that turned yellow were re-streaked onto LB agar plates containing apramycin and used to inoculate 2 mL LB broth cultures. Plasmid DNA was isolated and digested with *Bam*HI and *Hin*dIII to confirm the presence of the *xylE* gene in the correct orientation (Fig. 3.6A). Successful insertion of *xylE* was confirmed by the presence of an additional DNA band of 1.4 kb, which corresponds to the *xylE* gene, on the agarose gel when compared to similarly digested pAU230 (Fig. 3.6A; lanes 2 and 4). Construction of the *bldG* promoter probe plasmid was similar to that of pAU221 except that *xylE* was cloned directionally into pAU231 (Fig. 3.5B). The

Fig 3.5: Strategy used to insert *xylE* reporter gene into pAU230 and pAU231. (A) construction of helicase single copy promoter probe vector pAU221. Recombinant vector pAU230 was digested with *Bam*HI to accommodate the *xylE* gene, purified from pIJ4083, which harboured *Bam*HI and *Bgl*II sticky ends. Once *xylE* was inserted in the correct orientation (as shown), the helicase promoter probe construct was renamed pAU221. (B) construction of *bldG* single copy promoter probe vector pAU224. Recombinant vector pAU231 was digested with *Eco*RV and *Bam*HI to accommodate the *xylE* gene which was blunt on one end and which had a *Bgl*II cohesive overhang on the other end. Once the *xylE* gene was inserted in the correct orientation the recombinant plasmid was renamed pAU224. The thin black arrow shows the direction of *xylE* transcription.
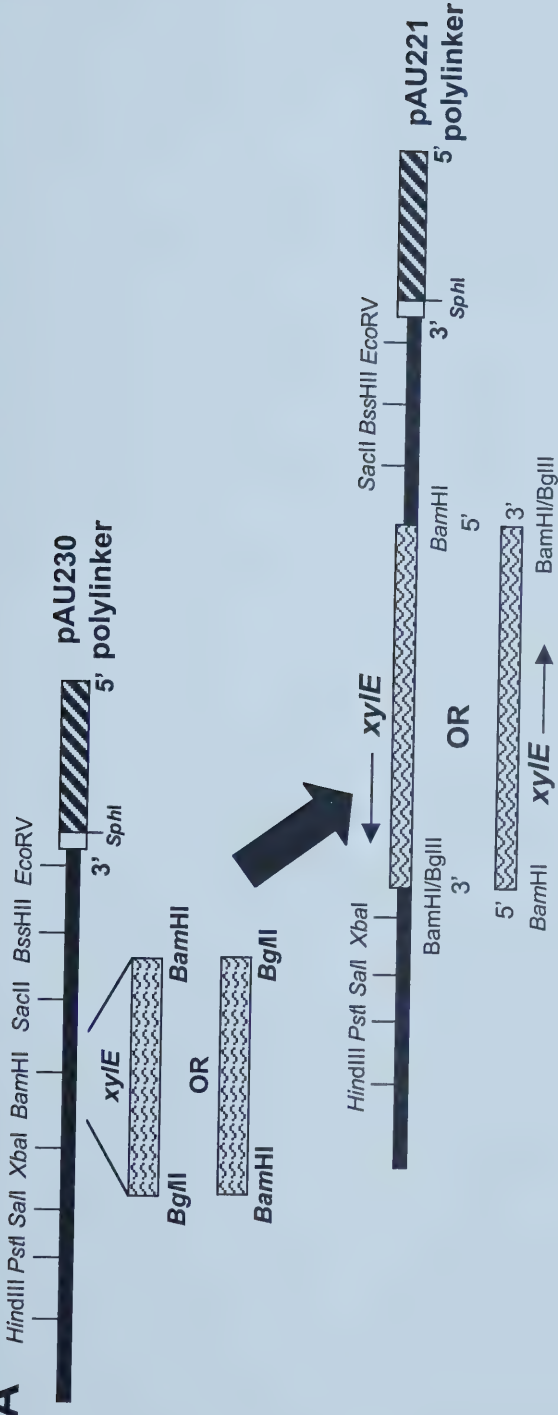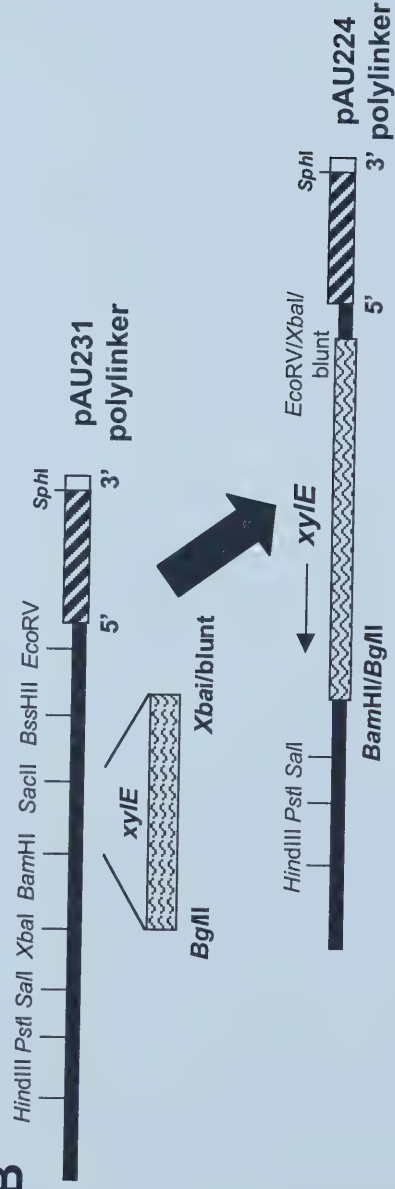
Figure 3.6: Verification of *xylE* insertion in pAU230 and pAU231 to generate pAU221 and pAU224. (A) 1% TBE agarose gel electrophoresis of the pAU221 plasmid DNA isolates digested with *Bam*HI and *Hin*dIII to confirm that *xylE* was present and in the correct orientation (see Fig. 3.4). The presence of a DNA band of ~1.4 kb (arrow with an asterisk; lanes 2 and 4) indicates successful insertion of *xylE* in the correct orientation. Samples were pooled for future use. (B) 1% TBE agarose gel electrophoresis of pAU224 clones digested with *Hin*dIII and *Eco*RV to confirm that *xylE* had been inserted in the correct orientation. Because of the loss of the *Eco*RV site upon ligation, no 1.4 kb DNA band was released. The DNA sample in lane 2 was assumed positive due to the larger band indicated by the spotted arrow. Presence and orientation of the *xylE* gene was confirmed by DNA sequencing.
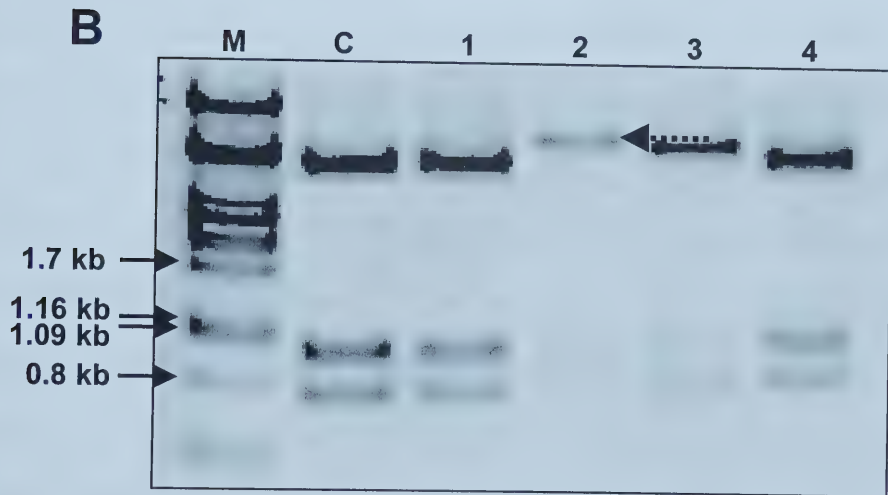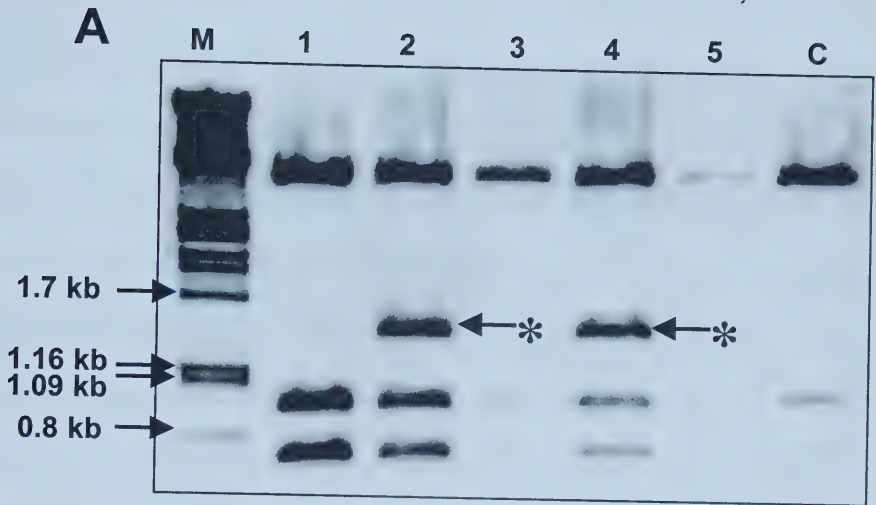
xylE gene was purified from pIJ4083 using *Xba*I digestion, blunt-ending of the digested DNA and then further digestion with *Bgl*II. This DNA fragment with one blunt-ended and one cohesive end, was ligated into pAU231 that had been digested with *Eco*RV (to generate a blunt end) and *Bam*HI (to generate a cohesive overhang compatible with *Bgl*II). The ligation mix was transformed into *E. coli* DH5α and four apramycin resistant colonies were recovered. Plasmid DNA was isolated from the recovered colonies and digested with *Hind*III and *Eco*RV, however, the recognition sequence for *Eco*RV was lost during ligation of the blunt-ended DNA ends and so the 1.4 kb *xylE* gene was not released. However, in Fig. 3.6B, the largest DNA band in lane 3 was found to be significantly larger than the largest band in the other lanes, suggesting that the recombinant contained the additional 1.4 kb DNA fragment. Therefore this recombinant plasmid was sequenced using JWA5 as primer, which anneals 140 nucleotides upstream of the *bldG* translation start. The results showed that the *xylE* reporter gene was ligated into the vector pAU231 in the correct orientation. The orientation of the helicase promoter and the *xylE* reporter gene was also confirmed by DNA sequencing using primer JST4, which anneals to the intergenic region DNA 30 nucleotides upstream of the proposed SCH5.13 ATG translation start codon. Each of the above sequencing reactions allowed determination of the sequence of the 3' end of the respective promoter DNA and the 5' *xylE* sequence, thus demonstrating that both insertions had occurred in the correct orientations. The single copy promoter probes for SCH5.13 and *bldG* were renamed pAU221 and pAU224, respectively.

After passage through the *dam⁻ dcm⁻ E. coli* ET12567 strain, the pAU221

and pAU224 plasmid DNA samples were used to transform *S. coelicolor* J1501.

Apramycin resistant colonies were re-streaked onto R2YE plates that contained

50 μg/mL apramycin. Four colonies of each of the pAU221 and pAU224

containing strains which maintained resistance to apramycin were patched onto

cellophane discs on R2YE agar containing apramycin. Before being used to

prepare full spore stocks of each of the strains, chromosomal DNA was isolated

from liquid cultures grown from each of the spore stock starting plates. This DNA

was used as a template for PCR using primers JST7 and JST8, which were

designed to anneal to regions of pSET152 flanking the multiple cloning site and

thereby amplify only DNA that had been cloned into this integrative vector (Fig.

3.7A). As shown in Fig. 3.7, amplified bands of 1.8 kb corresponding to the

promoter and *xylE* gene fusions cloned into pAU221 and pAU224 confirmed that

the plasmid had integrated into the chromosomal DNA. Spore stocks were

prepared using surface grown cultures that contained pAU221 and pAU224.

Only those colonies confirmed to contain the respective promoter probe vectors

were used to make spore stocks and perform quantitative *xylE* assays.

The amount of *xylE* gene product expressed in *S. coelicolor* J1501 strains

harbouring pAU221, pAU224 and pAU190 (see Table 2.3) was quantified from

cell free extracts that were prepared over a 48 hour time course. Plasmid

pAU190 was used as a negative control for *xylE* expression because this vector

contained no promoter capable of driving reporter gene expression. The results

of the assays concluded that while the *bldG* promoter (pAU224) could effectively

Figure 3.7: PCR amplification confirming the presence of pAU221 (B) or pAU224 (C) in the *S. coelicolor* chromosome. (A) pSET152 polylinker DNA sequence. Primers JST7 and JST8 allow for specific amplification of DNA cloned into the pSET152 polylinker. Restriction sites within the polylinker are shown. (B) and (C) Chromosomal DNA was isolated from apramycin resistant *S. coelicolor* colonies presumably containing either pAU221 (B) or pAU224 (C). This DNA was used as a template for PCR using primers JST7 and JST8. A band seen at ~1.8 kb corresponds to a 240 bp promoter region fused to the 1.4 kb *xylE* plus the pSET152 polylinker DNA. Lambda DNA digested with *Pst*I (lane M) was used for molecular weight determination. As a positive control, the original promoter probe plasmids (pAU221 or pAU224) were used as templates in identical PCR reactions (lane PC in A and B, respectively). Chromosomal DNA isolated from wild type *S. coelicolor* J1501 served as a negative control for the amplification (lane NC).

A

JST7 →

BamHI SacII BssHII EcoRV EcoRI HindIII PstI SalI XbaI

GGGTAACGCCAGGGTTTCCCAGTCACGACGGTTGTAAAACGACGGCCAGTGCCAAGCTTGGGCTGCCAGGTCGACTCTAG
CCCATTGCGGTCCCAAAAGGGTCAGTGCTGCAACATTTT GCTGCCGGTC ACGGTTCGAACCCGACGTCCTGCTGAGATC

-AGGATCC GCGGGCCGCGCGCGGGCGGGCGGGGCGGATATCGAATTCGTAATCATGTCAT AGCT GTT CCTGTGT GAAATT GTT ATCCGCTCACAAT
T CCT AGGCGCCGGCGCGCGCCGGCGCTATAGCTTAAGCATTAGTACAGTAT CGACAAAGGACACACTT T AACAATAGGCGAGTGTTA

← JST8

B

M PC NC 1 2 3 4

2.0 kb →
1.7 kb →

~1.8 kb

C

M 1 2 3 4 5 6 NC PC

2.0 kb →
1.7 kb →

~1.8 kb
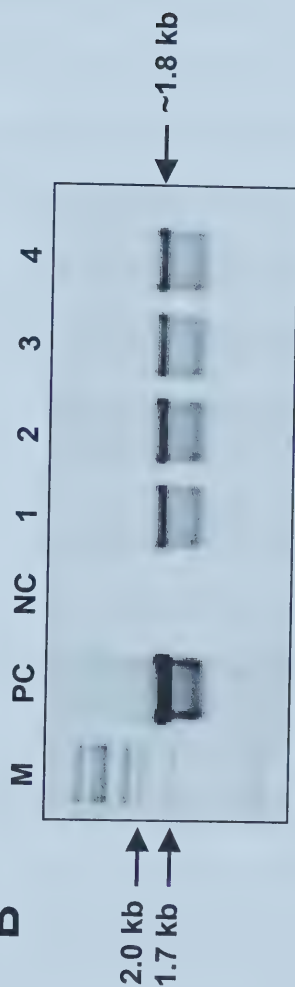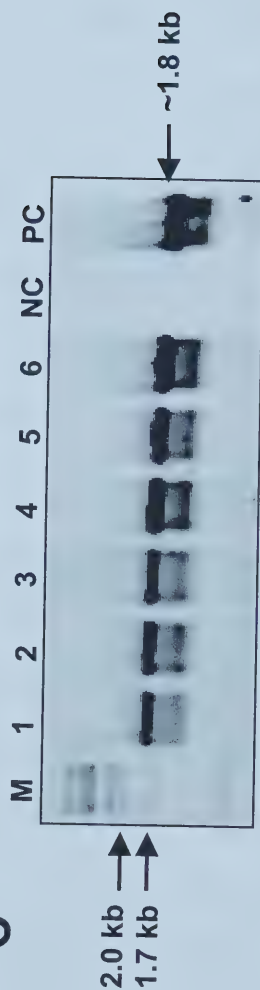
direct expression of the *xylE* reporter gene, the putative helicase promoter (pAU221), which is simply in the opposite orientation, was incapable of inducing detectable levels of the colourimetric compound 2-hydroxymuconic semialdehyde when compared with the negative control (pAU190). It should also be noted that throughout the entire course of sampling over the 48 hour time period, the strain harbouring pAU221 (with the putative helicase promoter driving *xylE* reporter gene expression) exhibited a delay in development of approximately six hours when compared to the other two strains. This was reproducible in two separate time course experiments using completely separate batches of medium each time. Why the presence of the putative helicase promoter in this orientation affected the growth of the strain remains to be determined.

3.1.4.2. Multi-copy expression using the high copy number vector pIJ4083

Because the single copy helicase promoter failed to direct *xylE* expression to detectable levels, the same promoter-containing fragment was ligated into the *Bam*HI restriction site of the *Streptomyces* promoter probe plasmid pIJ4083 (Clayton and Bibb, 1990) (Fig. 3.8A). This vector replicates in high numbers in *S. coelicolor* and so, although the copy number might vary with growth stage, the promoter activity should be amplified using this approach. This vector also contains the *tsr* gene for thiostrepton resistance, which can be used to select positive clones.

Since it lacks restriction / modification systems, *S. lividans* 1326 was used as the initial host for transformation with the ligated promoter probe plasmid.

Figure 3.8: Strategy used to construct the multiple copy promoter probe plasmids pAU222 and pAU233. (A) The helicase – *bldG* intergenic region was amplified using primers JST1 and JWA19 as described in Fig 3.2A. After digestion with *Bam*HI this 240 bp fragment was ligated into similarly digested pIJ4083 (restriction map shown; Clayton and Bibb, 1990). (B) Multiple cloning site of pIJ4083 is shown with the amplified DNA inserted. The intergenic region DNA could ligate in two possible orientations. The recombinant plasmid with the helicase promoter driving *xylE* expression was designated pAU222 and the plasmid with the *bldG* promoter driving *xylE* expression was designated pAU233.
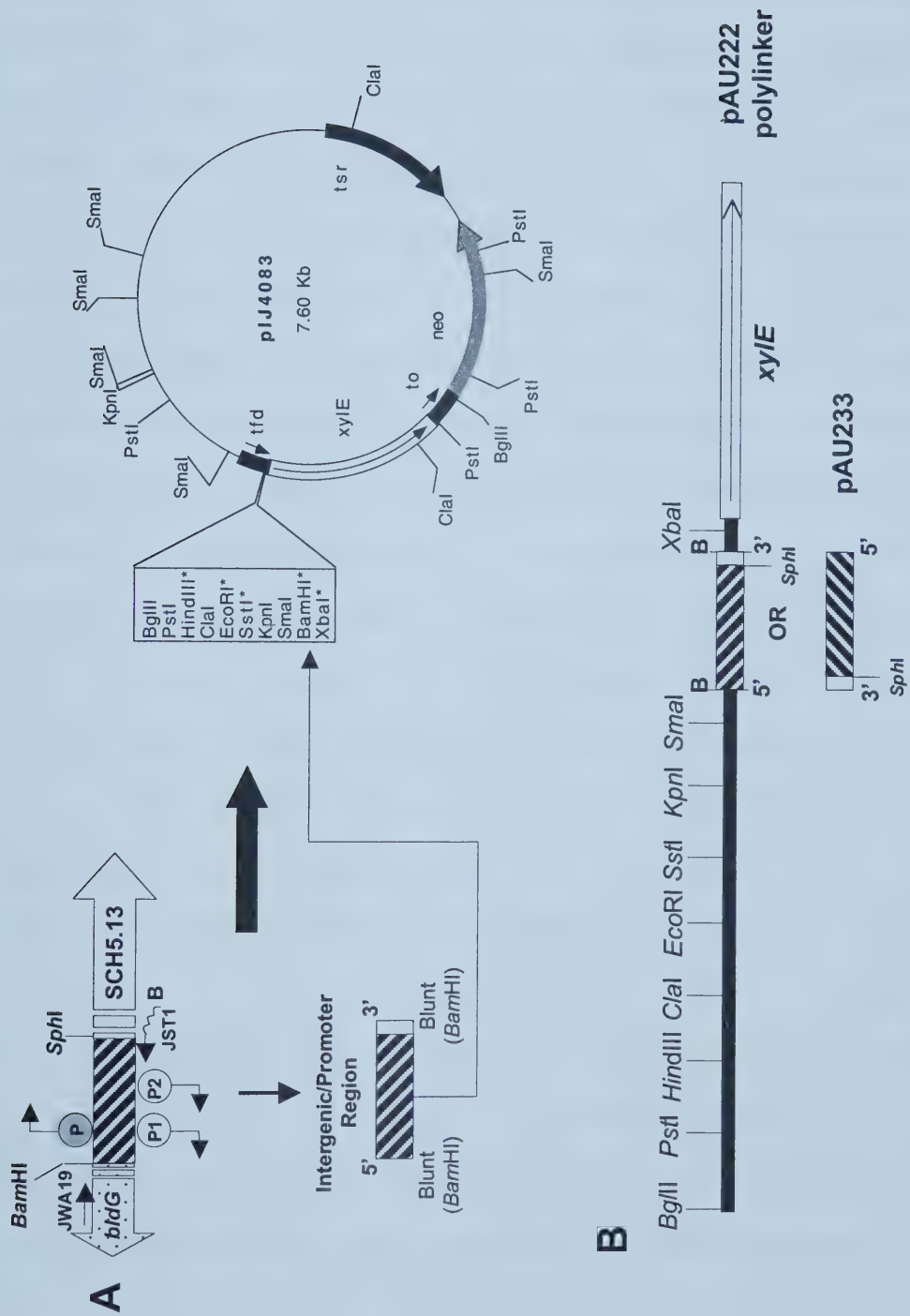
This strain was also ideal for screening purposes because *S. lividans* produces less of the pigmented antibiotics, actinorhodin and undecylprodigiosin, therefore transformants containing a cloned promoter will be visible as yellow colonies when sprayed with catechol. Thiostrepton resistant colonies were re-streaked onto R2YE agar plates containing 50 µg/mL thiostrepton. Those colonies that remained resistant to the antibiotic were then sprayed with catechol to look for the production of the yellow 2-hydroxymuconic semialdehyde. Yellow colonies were re-streaked again onto thiostrepton containing plates and were also used to inoculate trypticase soy broth (TSB) cultures containing 50 µg/mL thiostrepton. In order to determine if the helicase promoter had been successfully ligated into the multiple cloning site of pIJ4083 (Fig. 3.8), plasmid DNA isolated from the thiostrepton resistant colonies was amplified using the primers JST9 and JST10 (which were designed to anneal to the pIJ4083 multiple cloning site (Fig. 3.9)). The primers were expected to amplify a 420 bp fragment from the negative control, pIJ4083, and a 620 bp fragment if the promoter fragment was successfully inserted. A single clone that contained DNA inserted into the polylinker (Fig. 3.10A) was identified. Lane 4 on the figure shows the presence of an amplified band of a size consistent with insertion of the promoter fragment. To verify this, the plasmid DNA was further subjected to DNA sequence analysis using JST9 as the primer. The sequence revealed that the promoter was inserted in the orientation with *bldG* driving *xylE* (corresponds to the previously isolated pAU233 from above) rather than the helicase promoter driving *xylE*.

Figure 3.9: Multiple cloning site and flanking regions for vector pIJ4083.

Sequences flanking the multiple cloning site of pIJ4083 are shown in expanded

regions. Primers JST9 and JST10 were designed to anneal to specific

sequences in the terminator, tfd, sequence and the *xylE* DNA sequence. PCR

using these primers specifically amplified DNA that was cloned into the polylinker

of the vector.

tfd

BglII PstI HindIII ClaI EcoRI SstI KpnI SmaI BamHI XbaI

xylE

JST9

TAAACCGATACAATTAAAGGCTCCT TT TGGAGCCTTT TT TTT TGGAG
ATTT GGCTATGTTAATTT CCGAGGAAACCTCGGAAAAAAACCT C

JST10

ATGAACAAAGGTGTAATGCGACCGGGCCATGTGCAGCT GCGTGTACTGGACATGAGCAAGGCCCTGGAACACTACGT
TACT TGTT TCCACATTACGCT GGCCCGGTACACGTCGACGCACAT GACCTGTACTCGTT CCGGGACCTTGT GATGCA

Figure 3.10: Confirmation of successful cloning of the helicase – *bldG* intergenic region in pIJ4083. Thiostrepton resistant *S. lividans* transformants that turned yellow when sprayed with 0.5 M catechol were selected as possibly containing either the helicase or the *bldG* promoter probe vector. Plasmid DNA was isolated from the colonies and used as a template for PCR amplification using primers JST9 and JST10, which had been designed to anneal to pIJ4083 DNA that flanked its multiple cloning site. Molecular weight marker V (Roche; lane M in both A and B) was used for molecular weight determination. Lane numbers correspond to plasmid DNA isolated from separate colonies after growth in liquid culture. The parent plasmid pIJ4083 was used as a negative control template (lane C in both A and B). (A) The amplified DNA band of ~600 base pairs in lane 3 is consistent with the intergenic region DNA having been inserted into the pIJ4083 polylinker in *S. lividans*. This DNA was used to transform *S. coelicolor* and plasmid DNA was isolated from thiostrepton resistant transformants and subjected to PCR as described above. (B) The amplified band of ~600 bp in lane 1 confirms the presence of the promoter probe vector in *S. coelicolor*.
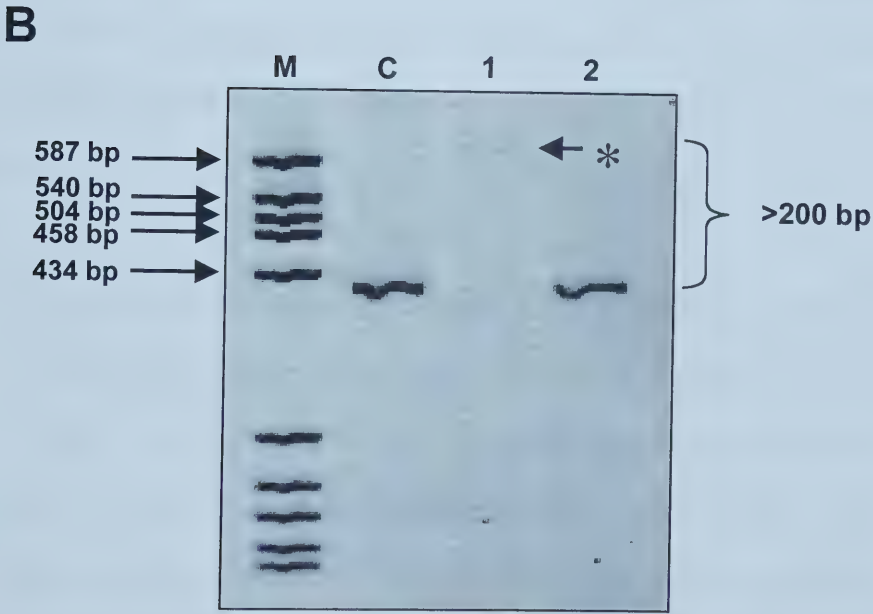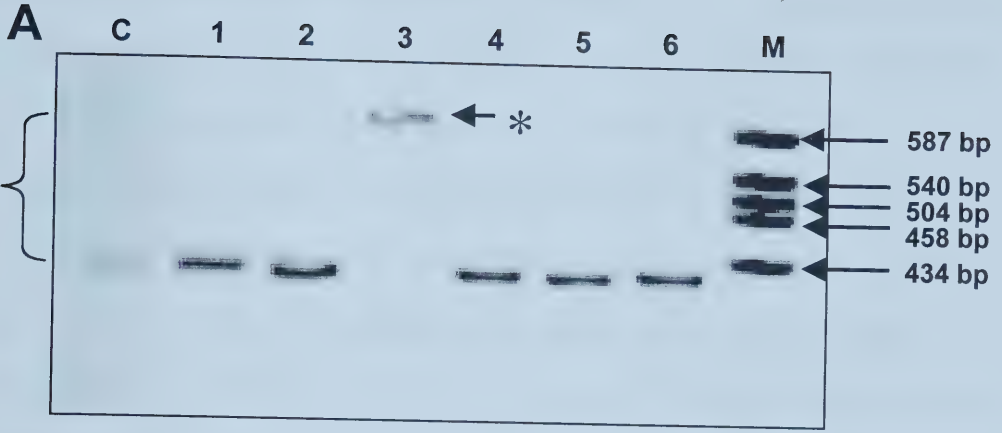
The *bldG* multi-copy promoter probe, pAU233, was used to transform *S. coelicolor* J1501 protoplasts. Five thiostrepton resistant colonies were isolated and remained resistant upon re-streaking. Plasmid DNA was isolated from one of the apramycin-resistant colonies and was used as a template for PCR using primers JST9 and JST10. The results in Fig. 3.10B confirmed that the *bldG* promoter DNA was present in the pIJ4083 polylinker, therefore pAU233 was successfully transformed into *S. coelicolor* and a spore stock of this new strain was prepared. Six additional independent attempts to isolate a recombinant plasmid with the intergenic DNA in the orientation that would allow the SCH5.13 promoter to drive *xylE* expression have failed. It is a possibility that multiple copies of this promoter are titrating away some unknown transcription factor that would result in an unhealthy situation for the organism, however it is unclear how the opposite orientation of the same fragment of DNA would have no adverse affects.

3.1.5. Use of reverse transcriptase polymerase chain reaction to quantify expression of the SCH5.13 putative helicase

Where they have been studied, some RNA helicases have been found to be expressed at low levels in the cell (Reuven *et al.*, 1995, Owttrim *et al.*, 1991). This could explain the difficulties experienced in visualizing the expression pattern of the SCH5.13 transcripts. Conventional RNA detection methods such as northern hybridization analysis, using either DNA or RNA probes, and S1 nuclease mapping were unable to detect the SCH5.13 RNA in total *Streptomyces*

RNA preparations. Reverse-transcriptase polymerase chain reaction (RT-PCR) involves the amplification of a cDNA synthesized using a thermostable reverse transcriptase (RT) (Fig. 3.11). The high temperature of the RT reaction (69°C) allows minimal interference by RNA secondary structure, an obstacle in all organisms but particularly in those with high G+C content, as is the case for *Streptomyces* species. Combining this efficient synthesis of cDNA with the subsequent amplification of the signal has allowed visualization of transcripts expressed at very low levels in both *Bacillus* (Hernandez *et al.*, 2000) and *E. coli* (Gill *et al.*, 1999). Therefore, this method was used in attempts to determine the expression pattern of the SCH5.13 – encoded helicase.

For these studies, the reverse transcriptase reaction was carried out using the oligonucleotide BKL87 as the primer and total RNA from *S. coelicolor* as the template. RNA used for these experiments was newly isolated so as to ensure the integrity of the template, which could be in question after a long storage period. BKL87 anneals specifically to the SCH5.13 mRNA (see Table 2.5; Fig. 3.12) template and should have allowed reverse transcription to proceed until the end of the template (i.e. the 5' end of the helicase transcript) was reached. The resulting cDNA was then used as a template for PCR using the end-labeled primers JST4 and BKL87, which anneal specifically within the SCH5.13 coding region. Samples of each completed reaction were electrophoresed on a 5% polyacrylamide gel that was subsequently dried and exposed to a phosphorscreen. In order to control for variances in RNA loading in each reaction tube, the RT-PCR procedure was also carried out using primers

Figure 3.11: Schematic drawing of reverse transcriptase polymerase chain reaction (RT-PCR). RNA harvested from surface grown *S. coelicolor* cultures was used as a template. An oligonucleotide primer (designated RT primer) with sequence specific to the complementary strand of the helicase gene anneals to the SCH5.13 mRNA transcript. Addition of a thermostable reverse transcriptase (designated RT) results in the synthesis of a cDNA which can be used as a template for PCR. Arrows indicate direction of synthesis of the primers. * indicates primer is radioactively labeled, resulting in labeling of the amplified DNA.

S. coelicolor → Total RNA

SCH5.13 mRNA

RT primer

RT

cDNA

Second strand synthesis

dsDNA

*

PCR
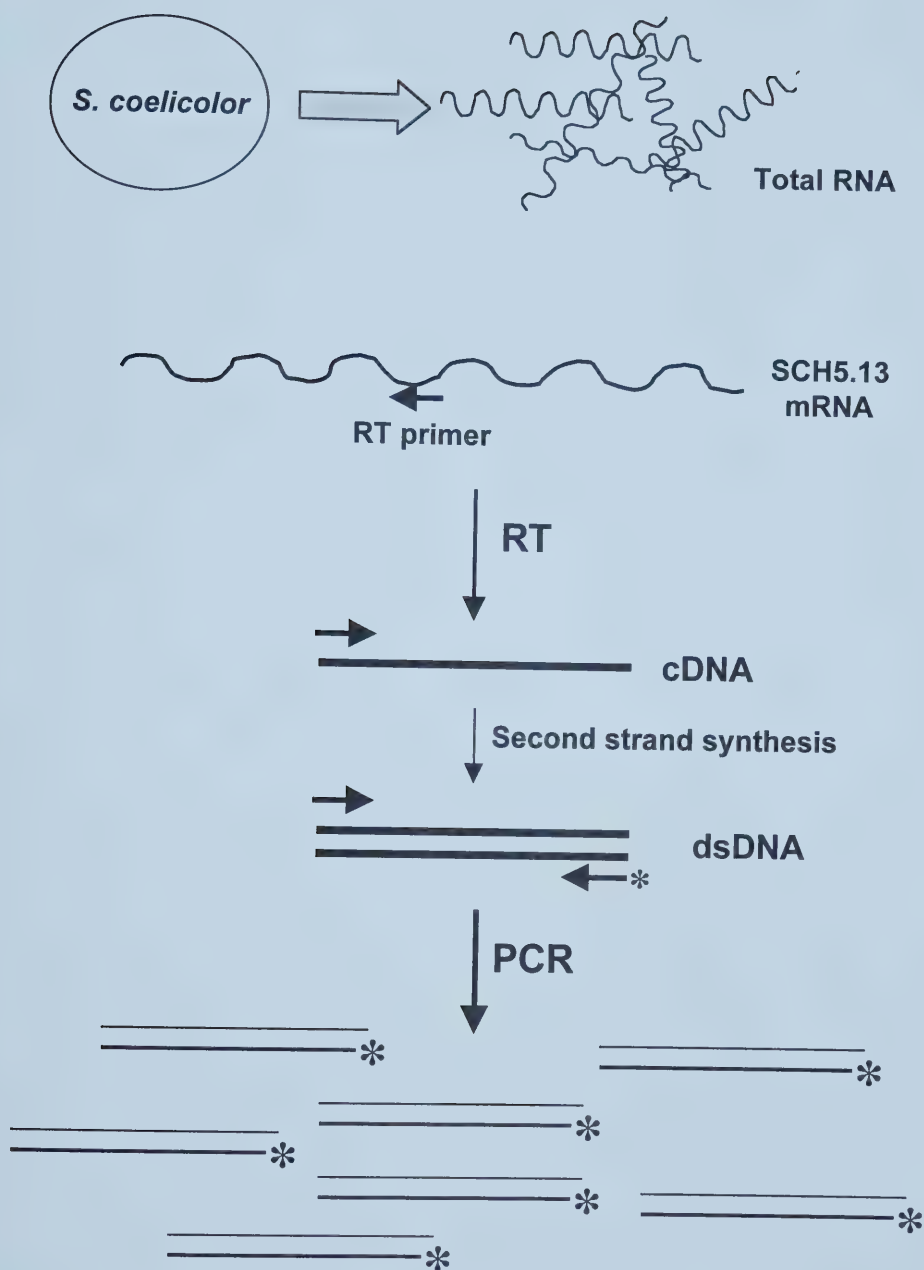
Figure 3.12: Determination of the expression pattern of the SCH5.13 –

encoded helicase using RT-PCR. Total RNA harvested at various times post

inoculation (shown in hours) was used as a template for RT-PCR. SCH5.13

transcription was assessed using the oligonucleotide BKL87 as the reverse

transcriptase (RT) primer and the cDNA was subsequently amplified using the

primers JST4 and end-labeled BKL87. As a control to ensure consistent RNA

template loading between samples, primers specific for *hrdB* (bottom panel) were

used on a duplicate aliquot from each time point sample. In this case, DBG7

served as the RT primer and DBG6 and end-labeled DBG7 were used for

amplification of the cDNA. (A) and (B) represent results from two independent

experiments using RNA isolated from two independent *S. coelicolor* time course

experiments. Using ImageQuant™ software the radioactivity in each lane of the

RT-PCR experiments (shown on the right) was quantified and corrected for RNA

loading levels to give transcript abundance (graph on left).

A

JST4 - BKL87
SCH5.13 transcript
DBG6-DBG7
hrdB control transcript

15hr 18hr 24hr 30hr 36hr 42hr 48hr

B

JST4 - BKL87
SCH5.13 transcript
DBG6-DBG7
hrdB control transcript

15hr 18hr 24hr 30hr 36hr 42hr 48hr
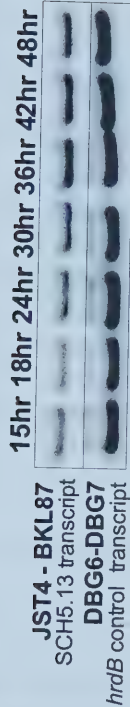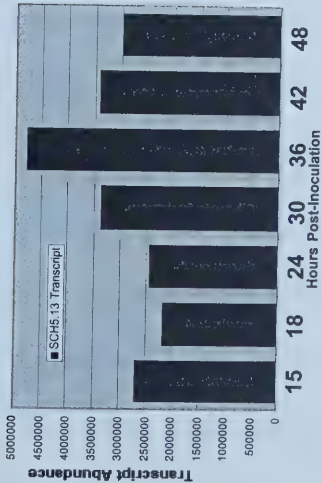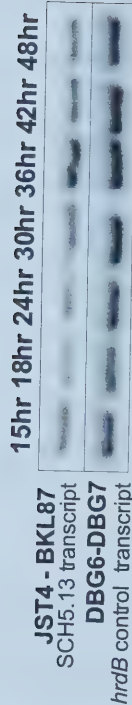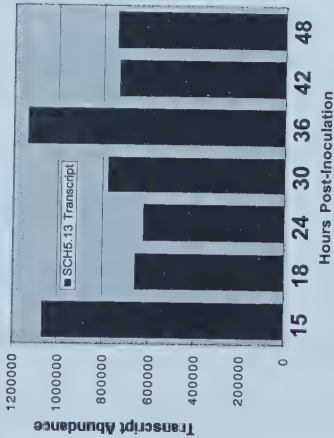
designed to amplify specifically the *hrdB* RNA transcript. *hrdB* encodes the main

σ^{70} – type sigma factor for *S. coelicolor* and is often used as a control because,

although it is not a highly abundant transcript, its expression is constitutive

throughout the life cycle (Kelemen *et al.*, 1996). Primers DBG6 and DBG7 were

designed (gift from D. Bignell, University of Alberta) for this purpose with DBG7

serving as the RT primer. The size of the amplified product was calculated using

a standard curve that had been constructed using the measurements traveled by

each band of known molecular weight in the commercial marker MWMV (Roche).

To ensure that the amplified products did not result from contaminating DNA in

the total RNA preparation, negative controls were included with experiments

performed using RNA from each independently performed time course. Samples

treated with RNAse consistently resulted in no amplification as did samples that

did not contain reverse transcriptase, confirming that the radioactive products

resulted from amplification of SCH5.13 mRNA.

Quantification of the helicase and *hrdB* transcripts by RT-PCR was

performed at least twice on RNA samples from each of three independent time

course experiments and representative results are shown in Fig.3.12. Each time

the experiment was repeated, the transcript for the RNA helicase was present at

much lower levels than the *hrdB* transcript, supporting the earlier suggestion that

low level expression might have precluded detection using the standard

methodologies. In addition, the putative RNA helicase transcript showed a

temporal pattern of expression with the high levels of expression at the onset of

sporulation (36 hour time sample). As expected, the *hrdB* transcripts were

constitutively expressed. When the bands from two independent experiments were quantified and corrected for RNA loading, the same growth-phase dependence was seen with high levels of SCH5.13 transcripts present at 15 and 36 hours post-inoculation (Fig. 3.12). The presence of a large amount of the SCH5.13 transcripts at the first harvesting time indicates a necessity for expression of this gene during vegetative growth. Therefore it seems possible based upon the transcription pattern, that this putative RNA helicase could play both a vegetative and a developmental role in the *S. coelicolor* life cycle.

3.2. Determination of the SCH5.13 Helicase Transcription Start Site

3.2.1. Primer extension analysis

Once the helicase expression pattern had been established as growth-phase dependent, it was important to determine where transcription of the gene initiated. Since earlier low resolution S1 nuclease protection assays had failed to even detect the helicase transcript, it was unlikely that high resolution S1 nuclease mapping would be useful in determining the transcription start site and so primer extension analysis was attempted. Primer extension analysis involves annealing an end-labeled oligonucleotide primer to the mRNA transcript (ideally within 200 nucleotides of the 5' end) of the gene of interest. Once annealed, the primer is used to initiate synthesis of a radioactive cDNA in the 3' to 5' direction, thus the resulting cDNA should terminate once the 5' end of the transcript is reached. To determine the transcription start site, the same primer is used to sequence the DNA gene of interest and is electrophoresed on the same

sequencing gel as the labeled cDNA. The nucleotide (as determined from the sequencing reaction) at which transcription initiates will co-migrate with the cDNA synthesized with the same primer.

In this case, to initiate reverse transcription, the oligonucleotide primer JWA0 which anneals to the RNA helicase sequence approximately 150 nucleotides downstream of the putative ATG start codon was added to a 40 µg aliquot of total RNA from the timecourse used previously for RT-PCR (Section 3.1.5). Annealing was allowed to take place for one hour at 37°C after denaturation for five minutes at 80°C. After reverse transcription was complete, separation of the reaction products on a 6% sequencing gel revealed no detectable bands. This procedure was repeated once more on the same RNA samples with the same results. A second alternative primer extension procedure (Gabriela Kelemen, personal communication) was then performed that included a slow cooling step between the denaturing and annealing steps and which used a different buffer system (see Section 2.4.6). Finally, this same procedure was attempted using the C. therm reverse transcriptase (Roche) and primer BKL87 because of the previous success in synthesizing cDNA for RT-PCR (Section 3.1.5). Again, with both attempts, no bands were observed suggesting, as was the case with S1 nuclease protection assays, that the mRNA was expressed below detectable levels.

3.2.2. Localization of the transcription start point using reverse transcriptase polymerase chain reaction

Since RT-PCR had proved sensitive enough to determine the expression pattern of the helicase transcripts, a series of experiments were designed to use this procedure to determine the approximate location the SCH5.13 transcript initiation site. Because this technique requires an amplification step and therefore the annealing of two primers within the transcribed sequence, RT-PCR cannot definitively identify the exact nucleotide that initiates transcription of the SCH5.13 ORF. Therefore, the strategy used here involved designing oligonucleotide primers that would "walk out" from the translation start site of the putative helicase gene towards the *bldG* ORF in order to narrow down the possible region of transcription initiation (Fig. 3.13A).

Before this part of the experiment was carried out, it was important to confirm that the transcription start site was within the SCH5.13 – *bldG* intergenic region and did not extend into the *bldG* coding sequence. Primers JST1 (which anneals to the SCH5.13 mRNA transcript at the proposed ATG translation start site and which was used for the RT step of the reaction) and JWA4 (which binds within the SCH5.13 - *bldG* intergenic region) were used to perform the RT-PCR procedure. If the SCH5.13 transcript initiated within the *bldG* coding region, then these two primers would be expected to yield an amplified band. As expected, there was no amplification across this region indicating that the end of the transcript was within the intergenic region. For the remaining experiments, it was important to keep the same primer for the RT step of the reaction so that any

Figure 3.13: RT-PCR strategy used to determine the transcription start site of SCH5.13. Starting at the 5' end of the SCH5.13 ORF, primers were designed to "walk out" towards *bldG* in order to narrow the possible region of transcription initiation for the putative helicase gene. (A) Schematic drawing (not to scale) of the helicase – *bldG* intergenic region showing the location of the previously mapped *bldG* promoters (Bignell *et al.*, 2000), and the location of the primers used to map the region of SCH5.13 transcription initiation. The primer names are given and are represented by arrows indicating their direction of synthesis as well as approximate region of homology with the cDNA. (B) RT-PCR amplification using various primer pairs. The RNA samples used were isolated at various times post-inoculation as indicated above the panels. Each set of reactions was carried out in parallel with *hrdB* as a control (shown in the bottom panel for each experiment).
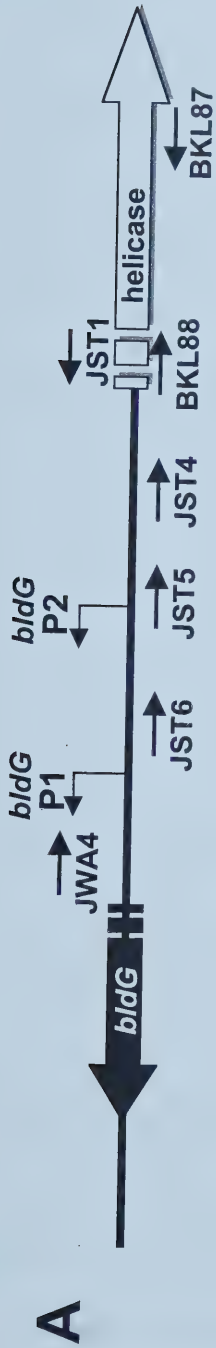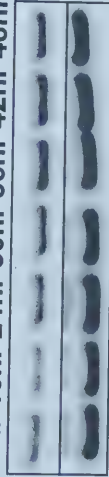
failure to amplify could not be attributed to failure to synthesize a cDNA in the first place. Because of its proven success in previous experiments (Section 3.1.5), oligonucleotide primer BKL87 was chosen as the RT primer. The results of the "primer walking" experiments are shown in Fig. 3.13B. As can be seen from the figure, use of either BKL88 or JST4 as the second primer for cDNA amplification resulted in easily identifiable bands, indicating that the transcript initiated upstream of JST4. When JST5 was used as the second primer only very weak bands could be observed, perhaps suggesting that JST5 was very close to the end of the transcript, thus causing difficulty in binding to the cDNA. Believing that the end of the transcript was close, JST6 was designed just 7 nucleotides upstream of JST5. Use of JST6 as the second primer resulted in no amplification of the cDNA, confirming that the transcription start site was somewhere within that primer binding sequence or between the JST6 and JST5 binding sites (Fig. 3.14). The size of all amplified products were calculated by comparison with Molecular Weight Marker V as described in Section 3.1.5 and all products agreed with the expected molecular weight to within +/- 5%.

Closer examination of the intergenic region sequence revealed that transcription of SCH5.13 initiates within the already characterized *bldG* promoter region (Fig. 3.14), strengthening the suggestion for a coordinate control mechanism for the expression of these two genes. Finally, the location of transcription initiation for the helicase gene as determined using RT-PCR strongly suggested that the GTG translation start codon proposed by the *Streptomyces* genome sequencing project (Fig. 3.1) is incorrect because the

Figure 3.14: DNA sequence of the SCH5.13 – *bldG* intergenic region. The double stranded DNA sequence of the intergenic region is shown with arrows representing oligonucleotide primers used in RT-PCR experiments. The arrows indicate both direction of synthesis and region of homology with the DNA. *bldG* – specific promoters and sequences are shown in blue while helicase – specific sequences are shown in pink. The helicase start codon predicted by the *Streptomyces* genome sequencing project (http://www.sanger.ac.uk/ Projects/S_coelicolor/) is shown in red, and the SCH5.13 start codon, together with a putative ribosome binding site (RBS), assigned as a result of these studies shown in Fig. 3.13 is shown in pink.

bldG start
codon

bldG
RBS

JWA4

CAGGGACAGG TCCACGGATC CTCCAGCACC TTGCTATCGA GCGGTCGTCCCTCGGGACACCTCGGCTTGA
GTCCCTGTCC AGGTGCCTAGGAGGTCGTGGAACGATAGCTCGCCAGCAGGAGCCCTGTGGAGCCGAACT

sequencing project
proposed start codon

JST6

bldG P2

bldGP1
-35

bldG P1

bldGP1
-10

AGCCCCCAGG ACGGTTCGCC AGCCGCGATGGCATTCAATCACTTACCGGCAGGCAGGCACGACGCCTTGG
TCGGGGGTCC TGCCAAGCGGTCGGCGCTACCGTAAGTTAGTGAATGGCCGTCCGCACGTGCTGCGGAACC

JST5

SCH5.13 start
codon

possible
RBS

BKL88

JST4

bldGP2 -10

TCCCATTGTC CGTCACGCCA GTGACAGACTCGGTGCCGATGGCCAAGAATCACCGACCCGATCGATCCCC
AGGGTAACAGGCAGTGCGGTCACTGTCTGAGCCACGGCTACCGGTTCTTAGTGGCTGGGCTAGCTAGGGGG

JST1

GGCGGAGCCCGGCTCCCGGCCGGAACCGGGCATGCTCCTGGACCGGCTCGCCGCGGGACCGGAGCGCGGGCT
CCGCCTCGGGCGCCGAGGGCCGGCCCTTGCCCGTACGAGGACCTGGCCGAGCGGCGCCCTGGCTCGGCCCGA

GCGCGGCATCA CTCATACGGA GCACTTGCCCCCGCGTGCGGGGCCGTCATGCCGTCTGGCCGGACCGGATTC
CGCGCGTAGTGAGTATGCCTCGTGAACGGGGGGGCGCACGCCCGGCAGTACGGCAGACCGGCCTGGCCTAAG

GGCCGGAGGTCCTGGCCGCGGTACGGGCGCGGGGCATCGAACATCCCTGGGCCCACCAGGCACGCGTGGC
CCGGCCTCCAGGACCGGCGCGCATGCCCGGCGCCCGTAGCTTGTAGGGACCCGGGTGGTCCGTGCGCACCG

BKL87

CGAGCACGCCCTGGACGACGGCGACTCGGTGGTCGTCGCCACCGGCACCGCGT
GCTCGTGCGGGACCTGCCGCTGAGCCACCAGCAGCGGTGGCCGTGGCGCA

translation start site is within the region proposed to be involved in transcription initiation. While leaderless mRNAs have been identified in *Streptomyces* (Janssen, 1993), they are rare. Furthermore, it was noted when performing amino acid sequence comparisons using Blast, that all similarities with proteins in the database began with a methionine that corresponded to an ATG codon further downstream from the GTG codon in the same reading frame which is preceded by a putative RBS (Fig. 3.14). This evidence suggests the more downstream translation initiation site would be used.

3.3. Creation of SCH5.13 Null Mutants

3.3.1. Use of wild type *S. coelicolor* J1501 parent background

In order to elucidate what role, if any, the putative RNA helicase plays in *S. coelicolor* development, it was necessary to attempt to construct a null mutant and observe any developmental consequences. There are two possible general strategies for gene disruption in *Streptomyces*, the single crossover strategy and the double crossover strategy. Double crossover gene disruption involves cloning two flanking regions of the gene in question (gene X) and inserting an antibiotic resistance gene (or other selectable marker) between them (Fig. 3.15A). The two flanking regions would initiate a double crossover into the chromosome via homologous recombination, thus replacing the wild-type gene X with the disrupted copy (illustrated in Fig. 3.15B). Complications can arise if the second crossover fails to occur (Fig. 3.15C) thus resulting in a false positive colony that is antibiotic resistant but also harbours a wild-type gene X. The

Figure 3.15: Schematic drawing of a double crossover gene disruption strategy. (A) *S. coelicolor* J1501 chromosome with a gene of interest (Gene X) shown as a spotted box. A suitable disruption vector is constructed where an antibiotic selection marker (checkered box) is inserted into the centre of the cloned Gene X. The flanking Gene X DNA promotes crossover into the chromosome via homologous recombination at (1), (2) or both. (B) Result of a double crossover into the chromosome. The Gene X coding sequence is interrupted with that of the antibiotic resistance gene. (C) Result of a single crossover at (1). Notice that there are now two copies of Gene X on the chromosome, one wild-type and one interrupted with the antibiotic resistance marker. The end result is a strain with wild-type phenotype that exhibits antibiotic resistance. The same outcome would result from a single crossover at (2).

A S. coelicolor chromosome

GENE X

1 2

Disruption Vector

C

Result of single crossover at 1

B

Result of double crossover
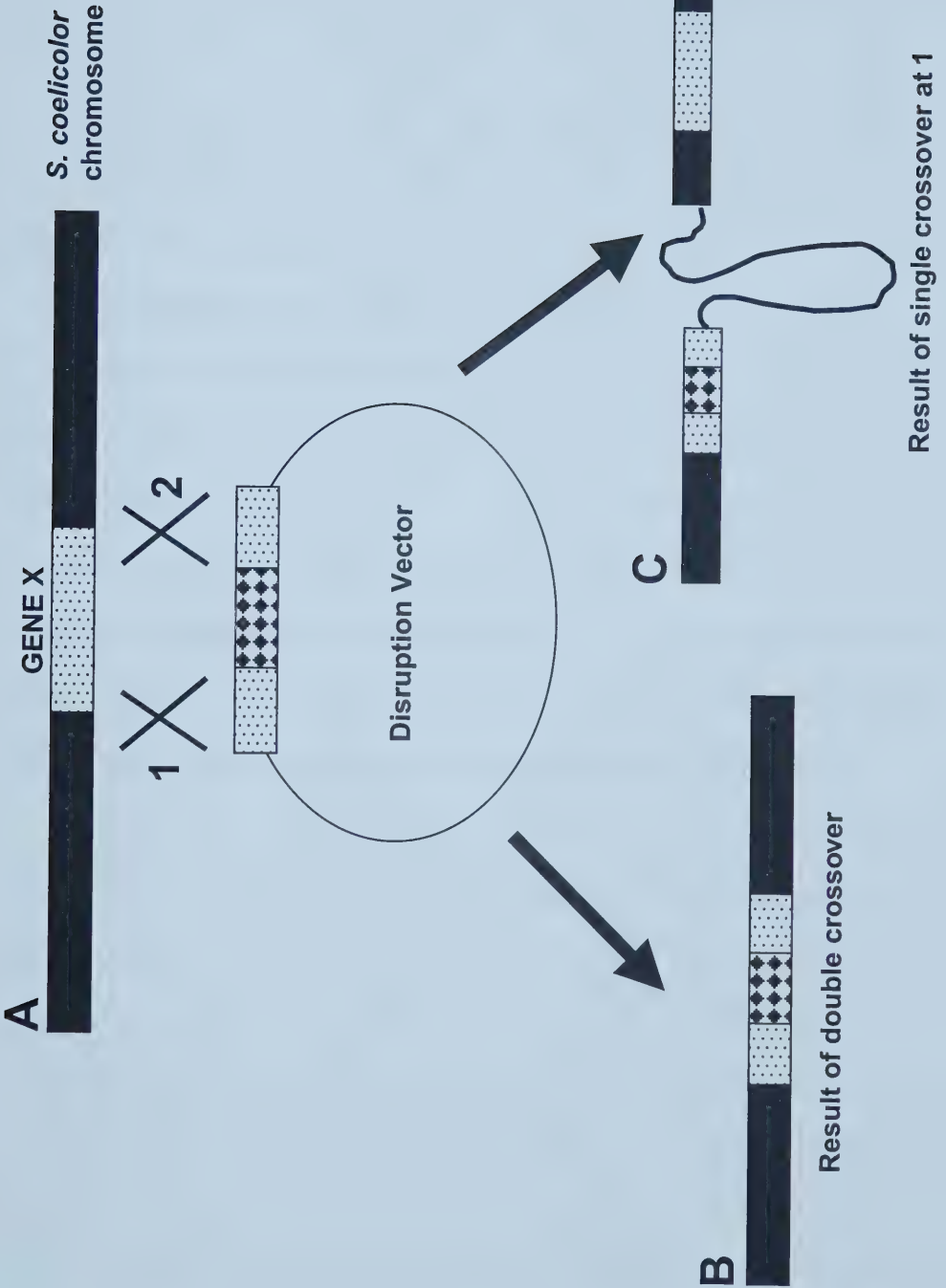
alternative is to use an internal DNA segment that will initiate a single crossover disruption. This strategy requires that the gene of interest be sufficiently large so that the internal DNA segment in question is long enough to initiate a cross-over by homologous recombination (>500 bp) yet still does not overlap the start or stop codons of the resulting protein. Given the size of the SCH5.13 ORF (~2.4 kb), it was decided that the single crossover strategy would be utilized in our attempts to construct a null mutant of the putative RNA helicase.

The complete strategy used in the construction of the necessary recombinant vectors is diagrammed in Fig. 3.16. A segment of the SCH5.13 helicase spanning nucleotides 417 to 1112 relative to the ATG translation start codon identified in Fig. 3.14 (~690 bp) was chosen as the internal DNA that would serve to initiate the single crossover. This crossover event would result in a truncated helicase gene terminating within the protein sequence that contains the DECH and SAT conserved motifs (Fig. 1.4), therefore leading to disruption within a region of the protein essential for helicase function (see Fig. 3.17).

First, the DNA was amplified from Cosmid H5 (a gift from H.M. Keiser). Cosmid H5 is one of an ordered set of cosmids created from high molecular weight *S. coelicolor* genomic DNA that had been partially digested with *Sau*3AI and ligated into Super*Cos*-1 (Redenbach *et al.*, 1996). Cosmid H5 has been completely sequenced by the *Streptomyces* genome sequencing project and contains 40 544 bp of the *S. coelicolor* chromosome. The SCH5.13 internal DNA sequence was amplified using primers BKL67 and JST2 (see Table 2.5) to give a product of 790 bp (see Fig. 3.16). This DNA was digested with *Bam*HI and *Kpn*I,

Figure 3.16: Construction of vectors pAU225 and pAU226 for use in helicase gene disruption. Using primers BKL67 and JST2 (see Table 2.5) a 790 bp region of DNA was PCR amplified using Cosmid H5 as a template. The primers are represented by black arrows indicating both region of homology with the DNA and direction of synthesis. The amplified DNA was digested with *Bam*HI and *Kpn*I (shown as B and K respectively), purified and the cohesive ends were blunt-ended. The blunt, 690 bp fragment was purified and cloned into the blunt-ended *Hin*dIII site of pIJ2925. The thiostrepton resistance gene was excised from pAU5 (Table 2.3) by digestion with *Xba*I and *Sma*I (shown as X and S, respectively). This 1100 bp fragment was ligated directionally into the *Xba*I / *Sma*I digested pAU225 to generate pAU226.

*Bgl*II *Pst*I *Hin*dIII *Sph*I *Sal*I *Xba*I *Bam*HI *Sma*I *Kpn*I *Sst*I,*Eco*RI *Bgl*II

pIJ2925 polylinker

BKL67

B

Cosmid
H5

K

JST2

PCR

B   K

Blunt
(*Hin*dIII/*Kpn*I)

Blunt
(*Hin*dIII/*Bam*HI)

*Bgl*II *Pst*I **690 bp helicase fragment** *Sph*I *Sal*I *Xba*I *Bam*HI *Sma*I *Kpn*I *Sst*I *Eco*RI *Bgl*II

**pAU225
polylinker**

X

pAU5

S

Blunt
(*Hin*dIII/*Kpn*I)

Blunt
(*Hin*dIII/*Bam*HI) *Xba*I

*Sma*I

*Bgl*II *Pst*I *Sph*I *Sal*I **Thiostrepton resistance gene** *Kpn*I *Sst*I *Eco*RI *Bgl*II

**pAU226
polylinker**

Figure 3.17: Single cross-over disruption strategy. The 690 bp SCH5.13 internal DNA fragment present in pAU226 should promote a single cross-over with the homologous region in the *S. coelicolor* chromosome. Upon selection with thiostrepton, the chromosomal helicase gene will be interrupted by pAU226, resulting in loss of helicase function.

SCH5.13

S. coelicolor
chromosome

B   K

pAU226

X

S

Single cross-over

Chromosomal SCH5.13
gene is disrupted.

*Bam*HI   *Kpn*I

= 690 bp SCH5.13 internal DNA
fragment

*Xba*I   *Sma*I

= thiostrepton resistance
gene (~1 kb)
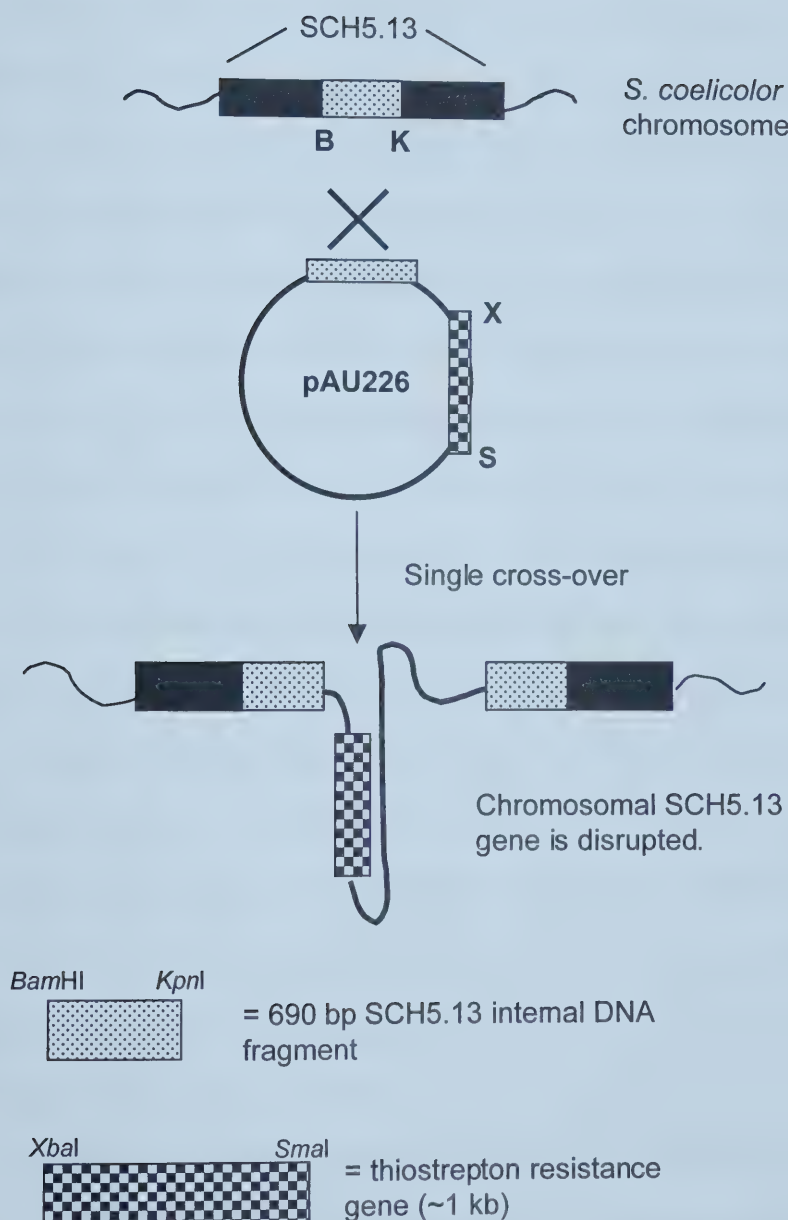
purified by crushing and soaking (Section 2.3.6) and blunt-ended. Plasmid

pIJ2925 was ideal as a parent vector for this project for many reasons. First, it is

a high copy number vector in *E. coli* with an ampicillin resistance cassette,

enabling initial cloning to be carried out in this organism instead of in

Streptomyces. Second, the multiple cloning site is within the lacZ' gene, which

allows blue/white selection for easy screening of cloned DNA. Finally, pIJ2925

cannot replicate in *Streptomyces*, therefore it must crossover into the

chromosome to be maintained. pIJ2925 was digested with *Hind*III and blunt-

ended to accommodate the 690 bp SCH5.13 DNA fragment that was previously

blunt-ended. *E. coli* DH5α, transformed with the ligation mixture containing

pIJ2925 and the helicase DNA, was spread onto LB agar containing ampicillin,

IPTG and X-gal, resulting in twelve white, ampicillin resistant colonies. Plasmid

DNA from these transformants was digested with *Bgl*II (whose recognition

sequences flank the multiple cloning site) to free any DNA fragments that had

been cloned into the pIJ2925 polylinker. If the SCH5.13 fragment had been

successfully cloned into pIJ2925, a DNA band of ~716 bp would be present in

the digest corresponding to 690 bp SCH5.13 DNA plus the 26 bp of polylinker

DNA. Upon digestion, six clones were found to contain the correct DNA

fragment (Fig. 3.18). Four of these plasmid preparations were pooled and this

new vector was renamed pAU225.

Thiostrepton was the antibiotic of choice to use to select for the crossover

into the *Streptomyces* chromosome, therefore the resistance gene, *tsr*, needed to

be cloned into pAU225. This was accomplished by digesting the plasmid pAU5

Figure 3.18: Confirmation of successful pAU225 construction. Plasmid DNA was isolated from 12 white transformants. The DNA was digested with *Bgl*II (see Fig. 3.16) to release the SCH5.13 internal DNA fragment that had been cloned into the pIJ2925 multiple cloning site. The resulting digested DNA was electrophoresed on a 1% TBE agarose gel, stained with ethidium bromide and visualized using a UV transilluminator. Lambda DNA digested with *Pst*I (lane M) was used for determining the chain length of DNA bands. The presence of a 716 bp DNA band corresponding to the 690 bp helicase fragment plus the pIJ2925 polylinker DNA (lanes 1, 3-6, 8), confirmed correct construction of pAU225. Samples 3-6 were pooled and kept for further manipulations.
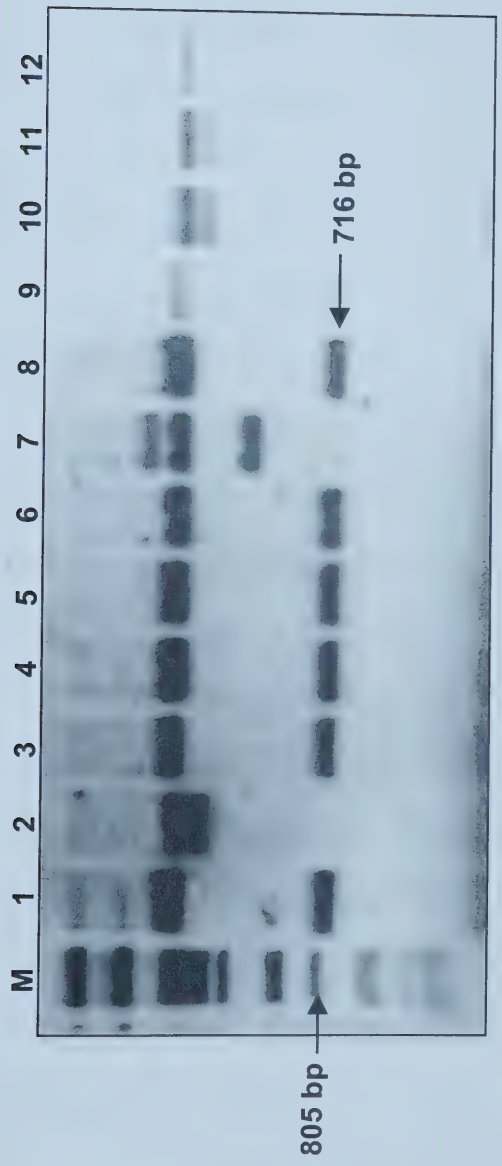
(Table 2.3) with *Xba*I and *Sma*I to free *tsr* and ligating it directionally into similarly

digested pAU225 (refer to Fig. 3.16). Thiostrepton cannot be used for antibiotic

resistance selection in *E. coli*, therefore, initial cloning in this organism was

performed using ampicillin, the resistance gene for which was also present in the

parent vector pAU225. Only three ampicillin resistant colonies were recovered

after transformation of *E. coli* DH5α. Plasmid DNA from these transformants was

isolated and digested with *Bgl*II, this time with the intent to reveal an increase in

the size of DNA released from the polylinker by the size of the *tsr* gene (1100

bp). Indeed when compared with pIJ2925 and pAU225 digested with the same

enzyme, the cloned DNA in the polylinker was of a length consistent with the 690

bp SCH5.13 DNA and the 1100 bp *tsr* DNA (Fig. 3.19). This recombinant vector

to be used in the disruption of SCH5.13 was renamed pAU226.

To achieve the proper methylation pattern necessary for successful

transformation into *S. coelicolor*, pAU226 was transformed into the *dam⁻*, *dcm⁻ E.

coli* strain ET12567. The plasmid DNA isolated from this strain was used to

transform *S. coelicolor* J1501. After selection was applied, recovery of any

thiostrepton resistant colonies was unsuccessful. The transformation was

repeated three more times, also with no success.

3.3.2. Use of the *S. coelicolor bldG* mutant strain as a host for putative helicase gene disruption.

In some cases, null mutants of genes required for development in

Streptomyces are only possible in sporulation deficient mutants as is the case

Figure 3.19: Confirmation of successful pAU226 construction. Plasmid DNA was digested with *Bgl*II to release DNA that had been ligated into the multiple cloning site of pAU225. The digested DNA was electrophoresed on a 1% TBE agarose gel, stained and visualized with a UV transilluminator. *Pst*I digested λ DNA (lane M) served as a guide for molecular weight determination of DNA bands. Lane 1 represents the parent vector pIJ2925 digested with *Bgl*II and served as a negative control. pAU225 digested with *Bgl*II (lane 2) was used as a control to show the size of the inserted DNA band. The presence of a band with a chain length of ~1800 bp in lane 3 confirms that the multiple cloning site contains both the 690 bp SCH5.13 internal DNA fragment and the 1100 bp thiostrepton resistance gene.

Lane labels: M 1 2 3

Right side (pointing to bands):
- 2700 bp — pIJ2925
- 1800 bp — Internal SCH5.13 DNA fragment + thiostrepton^R gene
- 716 bp — 690 bpSCH5.13 internal DNA fragment (+ flanking polylinker DNA)

Left side (marker sizes):
- 2840 bp
- 2577 bp
- 1980 bp
- 1700 bp
- 805 bp
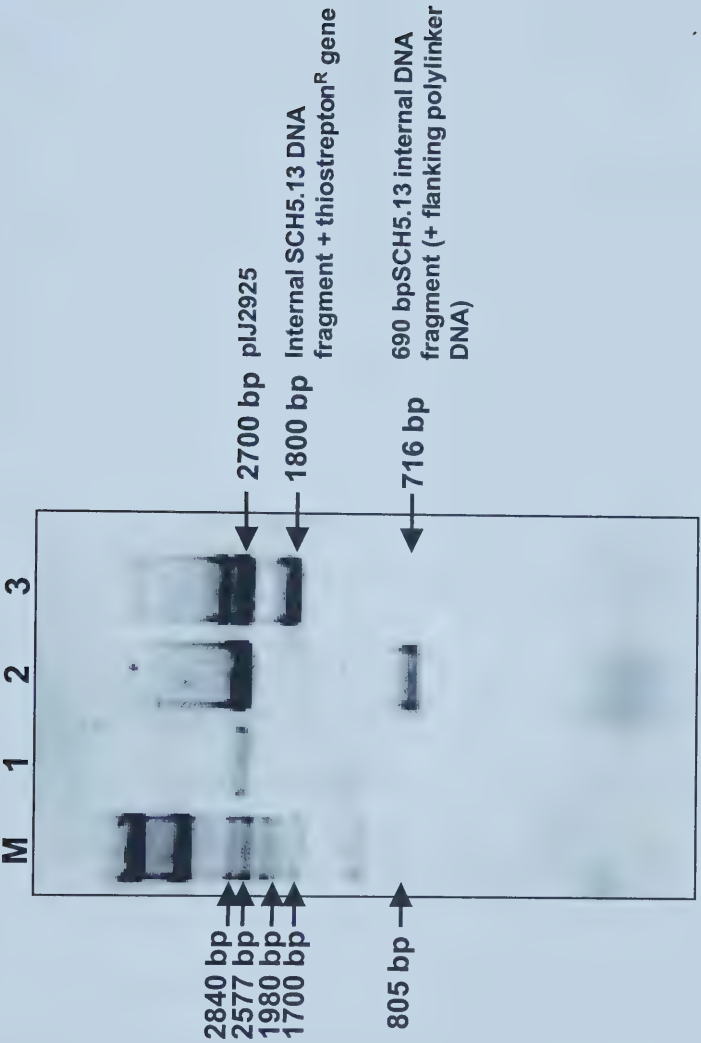
with the *S. griseus* gene, *nrsA* (formerly *orf1590*; McCue *et al.*, 1996). The

question was then raised, could SCH5.13 be disrupted in a *bldG* mutant? For

this purpose, *S. coelicolor* harbouring a *tsr* double crossover into *bldG* was used

as the host strain (a gift from Dawn Bignell, University of Alberta). The strategy

behind this disruption is diagrammed in Fig. 3.20. Because the *tsr* gene was

already present in the *bldG* mutant strain, a new putative helicase gene

disruption vector was needed with a different antibiotic selection marker. For this

purpose, apramycin was chosen and as such its resistance gene, Ap^R, was

ligated directionally into pAU225 after being liberated from vector pUC119ApR

 (Bignell *et al.*, 2000); Table 2.3) using *Eco*RI and *Bam*HI (Fig. 3.21). E. *coli*

DH5α was transformed with this ligation and positive clones were selected using

apramycin.

Plasmid DNA from one apramycin resistant colony was isolated and

digested with *Eco*RI and *Bam*HI to release the Ap^R gene from the polylinker.

This digest was compared with both pAU225 and pIJ2925 digested in a similar

manner (Fig. 3.22) to confirm insertion of the selectable marker DNA. This

recombinant plasmid was then renamed pAU229. Again, the proper methylation

pattern was obtained by passing the vector through *E. coli* ET12567 before being

used to transform the *S. coelicolor bldG* mutant, BldG3b (Bignell *et al.*, 2000).

Once transformed with pAU229, apramycin resistant transformants of

BldG3b were examined first for an altered phenotype (i.e. a change from the

characteristic 'bald' colony morphology) and, as expected, no change was seen.

The conclusion was that either disrupting the putative helicase gene in a *bldG*

Figure 3.20: Strategy for SCH5.13 disruption in a *bldG* mutant strain of *S. coelicolor.* (A) The 690 bp SCH5.13 DNA fragment present in pAU229 (see later) was used to initiate a single cross-over via homologous recombination into the *S. coelicolor bldG* mutant chromosome. The resulting disrupted chromosomal copy of the SCH5.13 ORF is shown in panel B. Chromosomal DNA was isolated from apramycin resistant transformants as well as from the parent *bldG* mutant strain. The DNA was digested with *Bam*HI and *Kpn*I to generate the fragments 1-4 shown in (B). To confirm the presence of these fragments, the digested DNA was separated by agarose gel electrophoresis and transferred to a nylon membrane by Southern transfer. A diagrammatic representation of the fragments that will be detected by hybridization analysis of this membrane with either the 690 bp SCH5.13 internal DNA fragment or the apramycin resistance gene is at the bottom of panel.

A

SCH5.13

S. coelicolor chromosome

BamHI KpnI

BamHI/HindIII
Blunt

KpnI/HindIII
Blunt

BamHI

pAU229

EcoRI

B

KpnI/HindIII
Blunt

BamHI

BamHI

KpnI

BamHI

EcoRI

BamHI/HindIII
Blunt

BamHI +
KpnI

1

2 = 716 bp

3 = 4.8 kb

3 = 4.8 kb

4

Probe with

Probe with

2 = 716 bp

3 = 4.8 kb

3 = 4.8 kb

= 690 bp internal SCH5.13
DNA disruption fragment.

= 1.4 kb apramycin
resistance gene cassette.
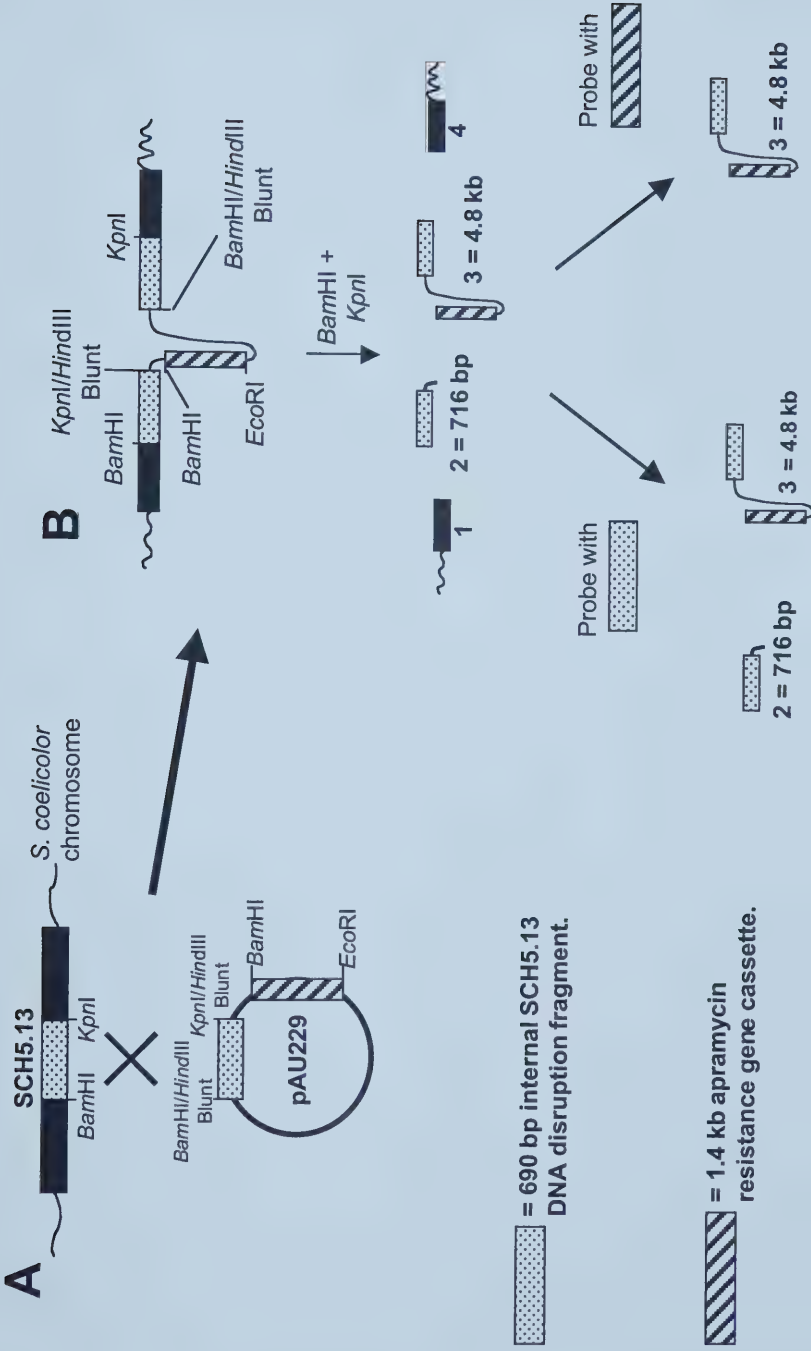
Figure 3.21: Construction of vector pAU229. The apramycin resistance gene was obtained by digesting pUC119ApR (Bignell *et al.*, 2000) with *Bam*HI and *Eco*RI (shown as B and E respectively). This 1400 bp fragment was gel purified and ligated directionally into the *Bam*HI / *Eco*RI digested pAU225.

pAU225
polylinker
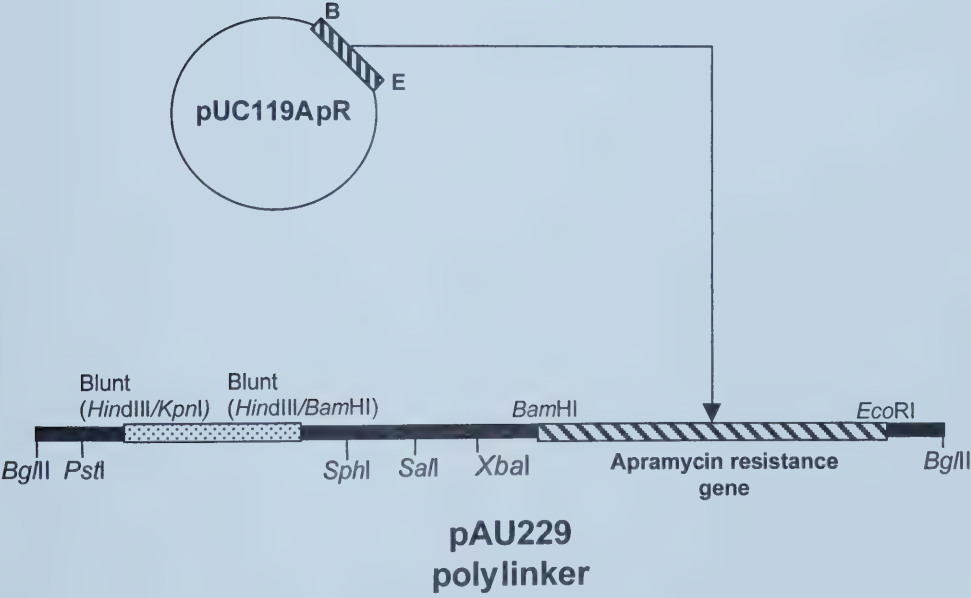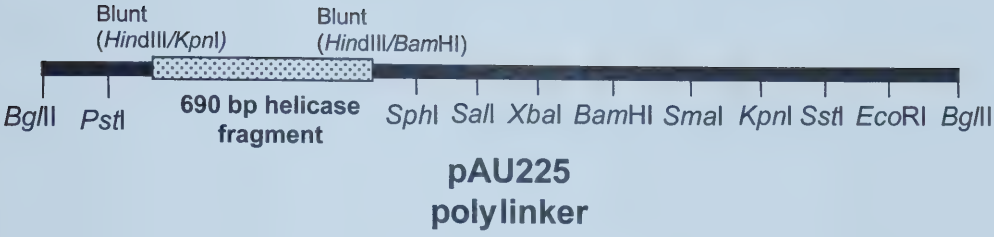
pAU229
polylinker

Figure 3.22: Confirmation of successful pAU229 construction. Plasmid DNA from an apramycin resistant transformant was isolated and digested with *Bam*HI and *Eco*RI to release the inserted apramycin resistance gene. The resulting digest was electrophoresed on a 1% TBE agarose gel (lane 3). λDNA that had been digested with *Pst*I was used as molecular weight marker (lane M). Negative controls included the vector pIJ2925 digested with *Bam*HI and *Eco*RI (lane 1), and the parent plasmid pAU225 digested with *Bam*HI and *Eco*RI (lane 2). The presence of a DNA band of approximately 1400 bp in length, corresponding to the size of the apramycin resistance gene, and a second band 3400 bp in length corresponding to the size of pAU225, confirmed that vector pAU229 had been successfully constructed.
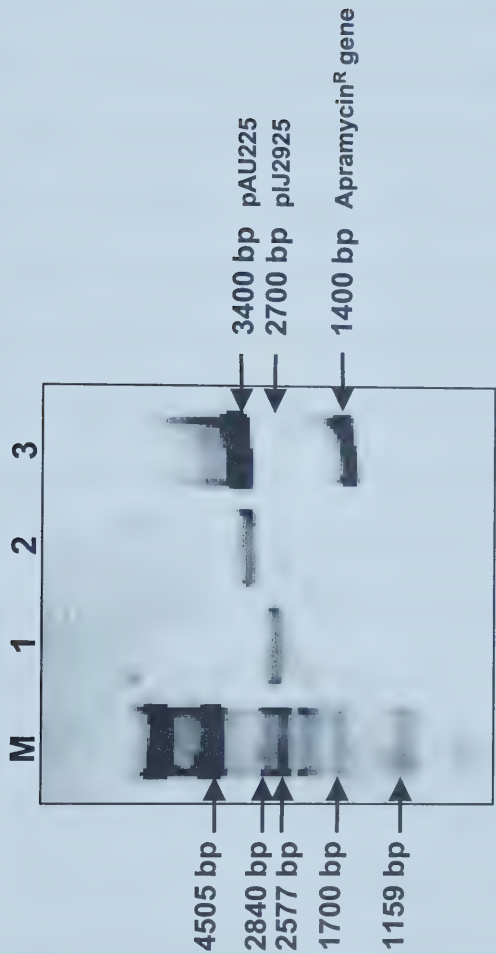
mutant had no apparent effect on phenotype, or that the apramycin resistant

colonies were the result of spontaneous resistance to this antibiotic.

To discount the latter conclusion, chromosomal DNA from the

transformants was isolated and digested with *Bam*HI and *Kpn*I. If the pAU229

was integrated into the chromosome, digestion with these enzymes would result

in four DNA fragments containing the SCH5.13 ORF sequence (shown in Fig.

3.20B). Two of the fragments would extend 670 bp upstream from the *Bam*HI

and 4983 bp downstream from the *Kpn*I into the flanking chromosomal DNA.

One fragment, corresponding to the 690 bp internal disruption sequence plus

polylinker DNA from pAU229, would be 716 bp in length and would be expected

to hybridize to the labeled 690 bp internal SCH5.13 helicase DNA probe. The

fourth fragment would also contain the 690 bp SCH5.13 internal sequence, but

would also contain the Ap^R gene, making it 4.8 kb in size. The digested DNA

was subjected to Southern hybridization analysis using random primer labeled

Ap^R fragment as a probe to determine if the gene was present in the

transformants. Indeed, a band of 4.8 kb was revealed in the transformant strain

and not in the control strain (Fig. 3.23A), strongly suggesting that this new strain

contained the integrated pAU229 and was deficient in both *bldG* and the

helicase. This idea was confirmed by stripping the membrane of radioactive

probe (Section 2.3.11) and subsequently re-probing the same membrane using

the 690 bp random primer labeled SCH5.13 helicase internal DNA fragment as

probe. The hybridization results were as expected with the chromosomal DNA

from the double mutant showing bands at ~716 bp and 4.8 kb, while the control

Figure 3.23: Confirmation of helicase disruption in a *bldG* mutant strain of *S. coelicolor.* Chromosomal DNA from a putative *bldG⁻*, SCH5.13⁻ double mutant and from the *bldG⁻* strain was processed for Southern hybridization as described previously (see Fig. 3.20). (A) Membrane probed with the apramycin resistance gene probe revealing a band of 4.8 kb corresponding to the size of the predicted fragment in the double mutant strain with pAU229 integrated into the chromosome. (B) The same membrane was stripped of labeled DNA probe and hybridized with the radiolabeled 690 bp SCH5.13 fragment revealing hybridization to a 690 bp fragment in the *bldG⁻* strain and the two expected fragments in the SCH5.13⁻, *bldG⁻* double mutant strain (see Fig. 3.20). Molecular weight marker III (lane M; Roche) was used for molecular weight determination.
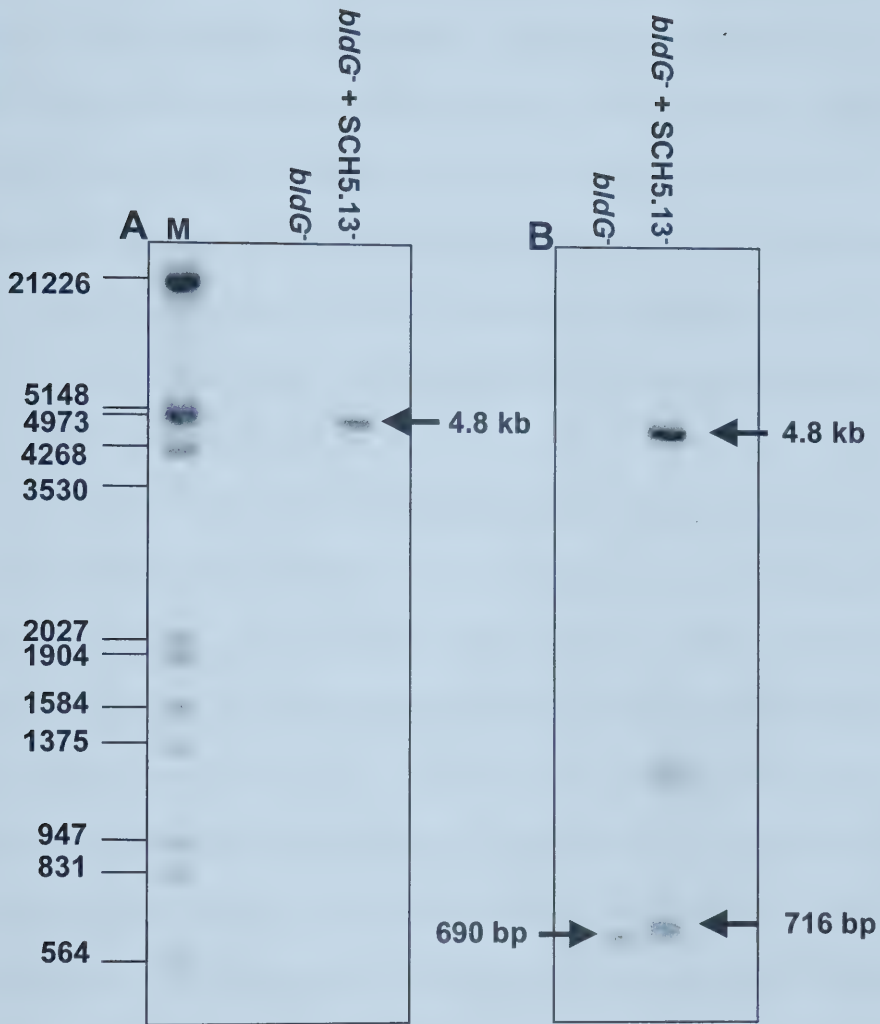
lane contained only one band corresponding to 690 bp (Fig. 3.23B). From these results we confirmed that a single cross-over disruption of the SCH5.13 helicase gene had occurred in a *bldG* mutant strain, and that this disruption did not result in any change from the 'bald' mutant phenotype. The fact that this disruption could be created in the *bldG* mutant but not in the wild type *S. coelicolor* suggests that the putative RNA helicase encoded by the SCH5.13 ORF could have a role, during vegetative growth, in suppressing gene(s) involved in sporulation, as is the case with *S. griseus nrsA*, and that early expression of those genes in the null mutant is lethal.

It should be noted that once these results were obtained, vector pAU229 was also used in further attempts to disrupt the helicase gene in wild-type *S. coelicolor*. Recall that use of pAU226, with the *tsr* marker rather than the Ap^R marker present pAU229, failed to allow isolation of a helicase mutant strain. After transformation of wild-type *S. coelicolor* with pAU229, apramycin was applied to the protoplasts for selection of a single cross over of the plasmid into the chromosome. As before, no antibiotic resistant colonies were recovered after three independent transformations, strengthening the belief that a null mutation of the helicase may not be possible in a wild type genetic background.

3.3.3. *S. coelicolor* with a second SCH5.13 gene integrated into the chromosome

The previous failure to recover any SCH5.13 null mutants in an otherwise wild type strain of *S. coelicolor* suggested that this helicase might be essential for

growth and that such a mutation would be lethal to this organism. To help

confirm this idea, a strategy to add an additional copy of the helicase SCH5.13

ORF to the *S. coelicolor* chromosome was devised (see below and Fig. 3.24).

The resulting strain could then be used as a host for transformation with the

disruption vector pAU226 (see above Section 3.3.2). If it was possible to isolate

transformants that contained pAU226 integrated into the chromosome in the

strain containing two copies of the SCH5.13 gene but not when a single copy of

the helicase gene was present, this would strengthen the suggestion that

creation of a null mutant is lethal.

　　　　To construct the 'double helicase' *S. coelicolor* strain, it was necessary to

clone the entire SCH5.13 ORF into the vector pSET152. pSET152 is an *E. coli*

shuttle vector that will integrate into the *S. coelicolor* chromosome at the φC31

phage attachment site and contains the apramycin resistance gene, Ap^R which

can be used as a selection marker in both *E. coli* and *S. coelicolor*. The simplest

method of cloning the SCH5.13 ORF into pSET152 was to first purify the entire

gene by digesting Cosmid H5 with *Nco*I and *Sst*I which resulted in the release of

a 3.1 kb fragment containing the entire helicase gene (nucleotides –332 to +2770

relative to the translation start site). The isolated DNA was first directionally

cloned into the pUC120 vector as diagrammed in Fig 3.25. This was done to

allow easier large scale purification of the SCH5.13 ORF than would occur if it

was from a cosmid which contains inserted DNA that is >40 kb in size. The 3.1

kb of SCH5.13 DNA would make up a small percentage of the cosmid but will be

~50% of the plasmid DNA when cloned into the 3.2 kb pUC120. Furthermore,

Figure 3.24: Strategy for SCH5.13 disruption in an *S. coelicolor* strain that has a second copy of the helicase gene in the chromosome. (A) Schematic drawing of the *S. coelicolor* chromosome and plasmid pAU228. The spotted box represents the SCH5.13 ORF (not to scale); * indicates the ϕC31 attachment site found both in the chromosome (exact location is not shown) and on the vector pAU228 used to initiate integration of the second helicase gene. (B) Once integration occurs, there will be a second copy of the SCH5.13 ORF within the chromosome and strains carrying the integrated DNA can be selected using apramycin resistance (Ap^R). Transformation of the resulting strain with disruption vector pAU226 and plating on thiostrepton – containing agar will select for single cross-over into one of the two SCH5.13 ORFs. The grey shaded region contained in pAU226 vector and in the middle of the full-length copy of SCH5.13, represents the 690 bp internal DNA sequence used to initiate homologous recombination. (C) The resulting chromosomal DNA after cross-over into either the native copy of the SCH5.13 gene or the second copy integrated at the ϕC31 *att* site.
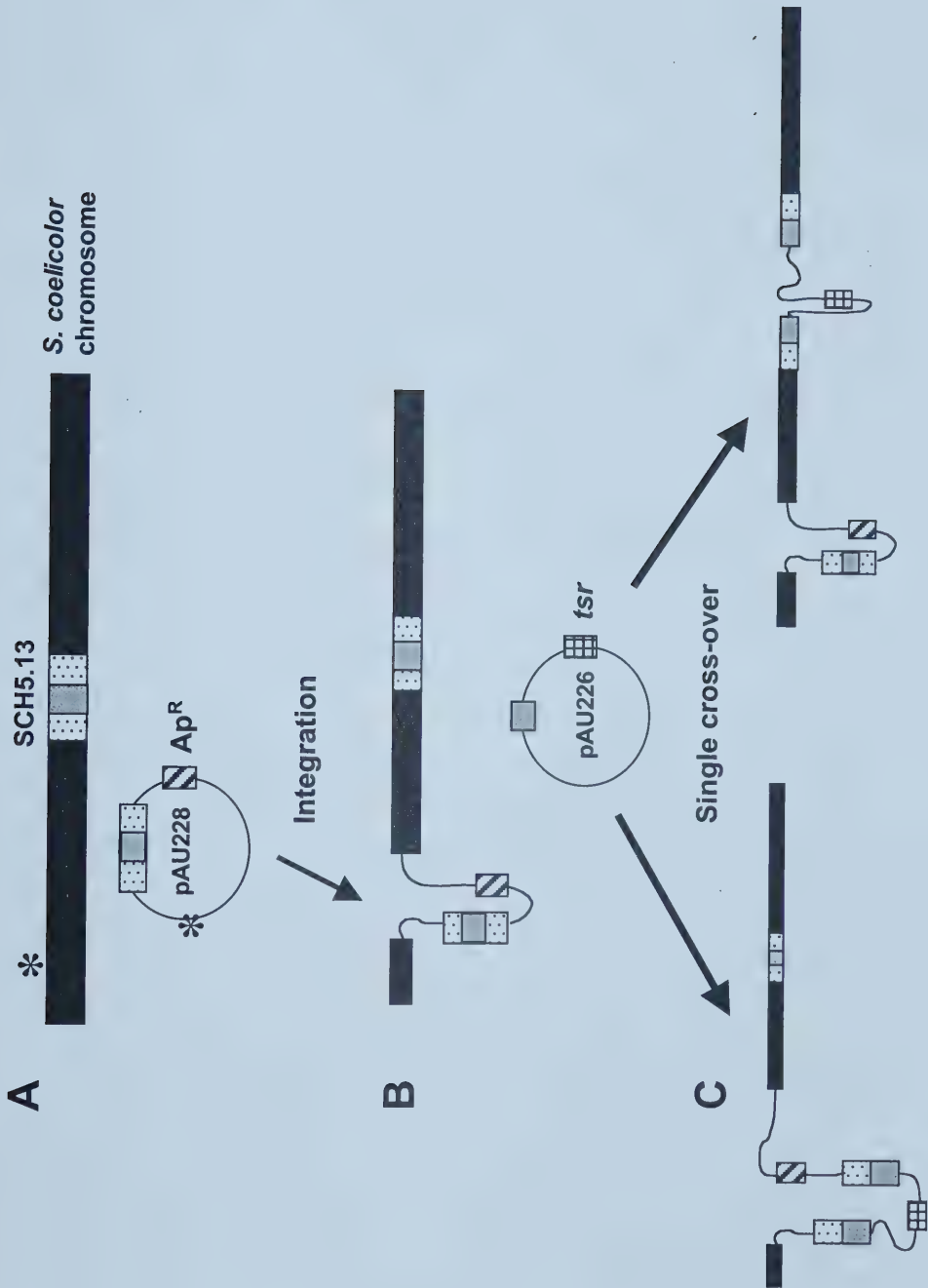
Figure 3.25: Construction of vector pAU227. A 3.1 kb *Nco*I / *Sst*I fragment from Cosmid H5 (Redenbach *et al.*, 1996) which contains the complete SCH5.13 ORF, was purified and ligated into the corresponding sites in the pUC120 polylinker.

Ncol BalI EcoRI SstI KpnI SmaI BamHI XbaI SalI PstI SphI HindIII **pUC120 polylinker**

SCH5.13 ORF (3.1 kb)

NcoI SstI

Cosmid H5 (~35 kb)

Ncol SstI KpnI SmaI BamHI XbaI SalI PstI SphI HindIII

SCH5.13 ORF (3.1 kb)

pAU227 polylinker
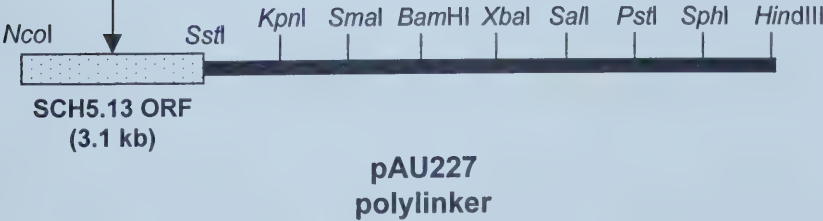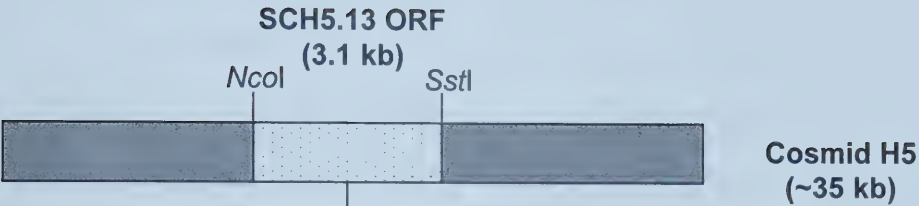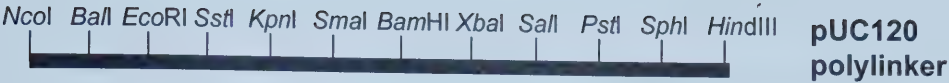
isolation of cosmid DNA from *E. coli* is difficult because of its large size while

pUC120 can be isolated easily by the alkali lysis method (Section 2.2.3).

Purification of the helicase-containing 3.1 kb fragment away from the *E. coli*

chromosomal DNA and from other cosmid H5 fragments was problematic

because of the low resolution of the agarose gel, and due to problems associated

with isolating a small piece of DNA from such a large vector.

Because of the possibility that a contaminating DNA fragment could have

been cloned into pUC120, colony hybridization was performed on 24 white,

ampicillin resistant colonies. The probe used was the random primer labeled 690

bp of DNA internal to the SCH5.13 coding sequence. Many hybridizing colonies

were identified (Fig. 3.26A) and plasmid DNA was isolated from four of the

positive colonies as well as two negative colonies as a control. The plasmid DNA

was digested with *Nco*I, *Sca*I and *Xba*I. *Nco*I and *Xba*I were used to free the 3.1

kb clone from the parent plasmid. However, pUC120 is very similar in size (3.2

kb) to the SCH5.13 DNA fragment and so a third enzyme (*Sca*I) was used to

digest the plasmid DNA into two fragments of approximately 1.5 and 1.7 kb. The

results shown in Fig. 3.26B confirmed that the 4 positive clones contained a 3.1

kb fragment of DNA, while the 2 negative controls contained DNA fragments

either too large or too small to be the helicase gene. All four positive plasmid

preparations were pooled and the new recombinant plasmid was renamed

pAU227. Further confirmation that pAU227 contained the correct SCH5.13 DNA

was achieved by sequencing using universal primer.

Figure 3.26: Confirmation of successful pAU227 construction. (A) After blue/white selection, white colonies were presumed to contain DNA in the pUC120 multiple cloning site. To determine if the cloned DNA was the SCH5.13 ORF, many white colonies were selected for colony hybridization in which the 690 bp SCH5.13 internal DNA fragment was used as the probe. Colonies 4, 5, 22 and 23 were selected as positive colonies while colonies 3 and 21 were selected as negative controls for further analysis. (B) Agarose gel electrophoresis of plasmid DNA isolated from the positive and negative colonies listed in (A), after digestion with *Nco*I, *Sca*I and *Xba*I to confirm that the DNA in the multiple cloning site was the correct size. λ DNA digested with *Pst*I (lane M) was used in molecular weight determination. pUC120 digested with *Nco*I, *Sca*I and *Xba*I (lane C) served as an additional control. The solid black arrow shows the 3.1 kb DNA band corresponding to the SCH5.13 helicase gene fragment (lanes 4, 5, 22 and 23) and the dashed black arrows show bands that are either slightly larger (lane 3) or lightly smaller (lane 21) than the expected size. The double-line arrow indicates partially digested pUC120 vector DNA.

A

B
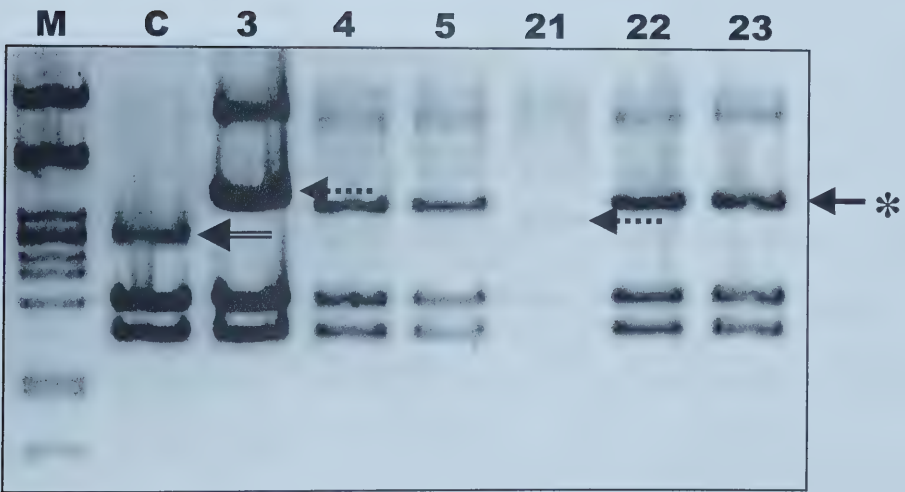
Recombinant vector pAU227 served as a useful tool for acquiring large amounts of pure SCH5.13 DNA. To purify the 3.1 kb SCH5.13 fragment for ligation into pSET152, pAU227 was digested with *Nco*I and subsequently blunt-ended. This linearized DNA was then digested with *Sca*I and *Xba*I, to liberate the helicase gene, and the fragment was purified using agarose gel electrophoresis. The DNA fragment was then ligated into the *Eco*RV / *Xba*I digested pSET152 polylinker (Fig. 3.27). White, apramycin resistant colonies were selected to further confirm the presence of the SCH5.13 gene in pSET152. Plasmid DNA from these colonies was digested with *Pst*I and compared to the parent vector pSET152 digested with the same enzyme (Fig. 3.28). Successful ligation of the helicase gene fragment into the multiple cloning site, resulted in an increase in size of the ~2.3 kb pSET152 DNA band to ~5.4 kb. Indeed, a number of the potential positive clones did possess a 5.4 kb band and were pooled. The recombinant vector was renamed pAU228.

After passage through *E. coli* ET12567, recombinant vector pAU228 was used to transform *S. coelicolor* J1501 in order to supply the second copy of the SCH5.13 gene. Selection for transformants involved overlaying protoplasts with apramycin to a final concentration of 50 µg/mL. A number of resistant colonies were recovered and remained apramycin resistant when re-streaked onto R2YE agar plates containing 50 µg/mL apramycin.

Interestingly these colonies displayed an altered phenotype when compared with the wild-type *S. coelicolor* J1501 even when grown without antibiotic selection (Fig. 3.29). The strain exhibited a delay in development of

Figure 3.27: Construction of vector pAU228. The 3.1 kb SCH5.13 fragment (see Fig. 3.25) was excised from vector pAU227 by first digesting the plasmid with *Nco*I and then blunt-ending the DNA. The linearized vector was then digested with *Xba*I and *Sca*I to liberate the SCH5.13 DNA. This *Xba*I – blunt DNA fragment was purified using agarose gel electrophoresis before being ligated into pSET152 digested with *Eco*RV and *Xba*I.

HindIII   PstI   SalI   XbaI   BamHI   SacII   BssHII   EcoRV   EcoRI

pSET152 polylinker

NcoI   SstI   KpnI   SmaI   BamHI   XbaI   SalI   PstI   SphI   HindIII

SCH5.13 ORF (3.1 kb)
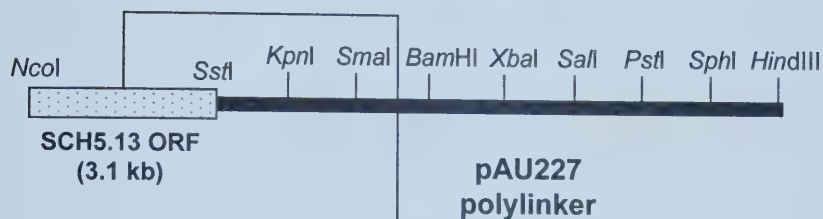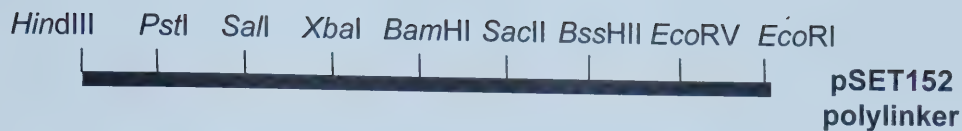
pAU227 polylinker

NcoI -blunt + XbaI

NcoI / blunt   XbaI

SCH5.13 ORF (3.1 kb)

+ EcoRV / XbaI digested pSET152

HindIII   PstI   SalI   XbaI   NcoI/blunt EcoRV   EcoRI

pAU228 polylinker with SCH5.13 insert

Figure 3.28: Confirmation of successful pAU228 construction. Plasmid DNA from 4 white, apramycin resistant colonies was isolated and digested with *Pst*I to determine whether the SCH5.13 ORF had been successfully ligated into pSET152. The parent vector pSET152 digested with *Pst*I served as a control (lane C). λDNA digested with *Pst*I (lane M) was used for molecular weight determination. The dotted arrows denote the vector bands and the solid arrow indicates a 5.4 kb band that corresponds to 2.3 kb of pSET152 DNA plus the 3.1 kb of helicase DNA cloned into the vector.
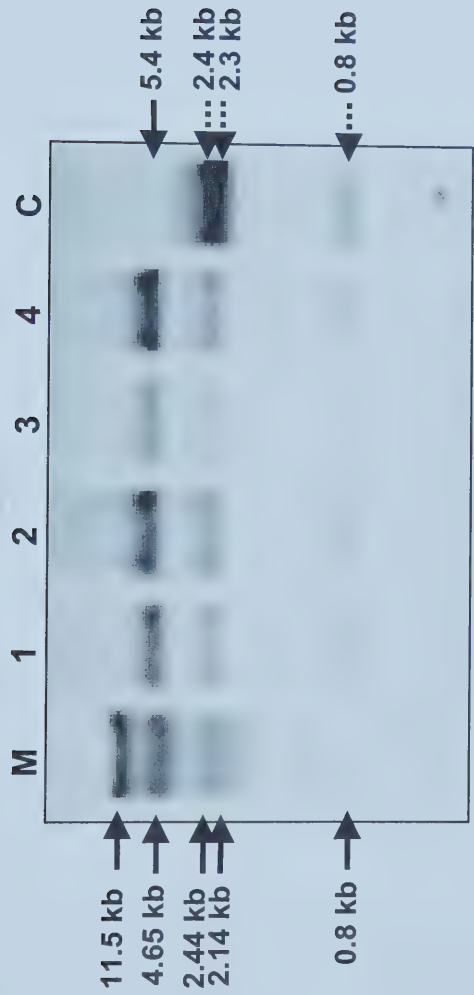
Figure 3.29: *S. coelicolor* J1501 colonies with (B) and without (A) an additional copy of the SCH5.13 gene integrated into the chromosome. The entire helicase gene was cloned into pSET152, an integrative shuttle vector that integrates into the chromosome at a phage attachment site, creating recombinant vector pAU228. Transformation of *S. coelicolor* J1501and selection for apramycin resistance (the selectable marker on pSET152) allowed isolation of a strain containing 2 copies of the helicase. The strains were patched onto R2YE agar and photographed after 72 hours of growth.

A

B

S. coelicolor J1501   *S. coelicolor* J1501 + pAU228

approximately 12–15 hours when compared to the wild-type strain of *S. coelicolor*. Further examination of this strain has been problematic as it has been incapable of growth in liquid culture despite utilizing a number of different growth media. Consequently, producing protoplasts of this strain has been impossible. Since protoplast transformation is the preferred method of introducing the gene disruption plasmid into the strain, disruption of the helicase could not be carried out.

Chapter 4:

Discussion

4. DISCUSSION

RNA helicases are a group of enzymes categorized within a large superfamily of nucleic-acid associated proteins that utilize energy derived from ATP or other nucleoside triphosphates to unwind RNA duplexes. Such proteins have been implicated in numerous cellular processes in which mRNA secondary structure is deleterious to survival of the organism including translation initiation, cold shock response, mRNA turnover and development. So far RNA helicases have been identified in all branches of life including viruses, bacteria, eukaryotes and archae, however until now there have been no known RNA helicases identified in the multicellular prokaryote *Streptomyces*.

The putative RNA helicase, SCH5.13, was first identified as a partial ORF during the initial sequencing of the *bldG* locus. The complete sequence of this gene was obtained later from the *Streptomyces* genome sequencing project database (Cosmid H5 AC# AL035636), and it was confirmed that the resulting amino acid sequence greatly resembled other known and hypothetical RNA helicases including the well-characterized eIF-4a (Gorbalenya *et al.*, 1989). The putative RNA helicase encoded by SCH5.13 contains many of the conserved amino acid motifs characteristic of RNA helicase proteins including the modified DEAD-box sequence, DECH (asp-glu-cys-his) which is present in many RNA unwinding proteins required for development and differentiation. Scrutiny of the DNA sequence for the putative RNA helicase, SCH5.13, noted that there was a possibility that the putative GTG start codon identified by the *Streptomyces* genome sequencing project database was incorrect as no reasonable ribosome

binding site could be identified. Using FRAME analysis, a putative ATG start codon was identified 117 nucleotides downstream of the GTG that corresponded to the methionine previously noted to begin all sequence similarity between SCH5.13 and other RNA helicases according to Blast search results. A plausible ribosome binding site (GGAA) was identified six nucleotides upstream from the ATG codon as well as a potential –10 promoter sequence (TTCAAT), however there was no readily identifiable –35 recognition sequence showing similarity to previously characterized *Streptomyces* promoters (Strohl, 1992). The absence of a consensus –35 region is not uncommon in *Streptomyces* as several promoter sequence compilations have noted numerous promoters lacking obvious –35 promoter recognition sequences (Strohl, 1992; Bourn and Babb, 1995).

Further confirmation that the ATG codon was the more reasonable start codon was obtained using RT-PCR to map the start of transcription for the SCH5.13 ORF. No amplification of the SCH5.13 transcript occurred using an oligonucleotide primer that overlapped this GTG codon. Presumably the lack of amplified signal was due to the 5' end of the SCH5.13 transcript occurring prior to this sequence, thus making it nearly impossible for this GTG codon to initiate translation. However, with no ability to clearly define the exact site of transcription initiation, it cannot be ruled out that the putative RNA helicase SCH5.13 is translated from a leaderless mRNA as has been noted, although rarely, in other *Streptomyces* genes (Janssen, 1993). To clearly determine which of the two potential start codons discussed is the true site for translation initiation

it would be useful to perform site directed mutagenesis on both the GTG codon indicated by the genome sequencing project, and the ATG codon that has been identified in this study. If a mutation in the ATG codon resulted in aberrant translation of the helicase while mutating the GTG codon had no effect on the expression of the SCH5.13 gene product, then it would be reasonable to conclude that indeed the ATG codon that has been identified and discussed throughout this thesis project is the start codon for protein translation.

In order for the site directed mutagenesis of the two potential start codons to be useful, it will also be necessary to purify the protein product expressed from the SCH5.13 ORF. Experiments are underway (J. Stoehr, K. Gislason and B.K. Leskiw, unpublished) in which the entire SCH5.13 ORF will be cloned into the pMAL overexpression vector. The SCH5.13-encoded protein will be expressed in *E. coli* as a fusion with maltose binding protein, which can be easily purified using column chromatography. Antibodies can then be raised against the protein and its expression pattern can be determined by western analysis both in the mutagenized strains as well as the wild type. Detecting the SCH5.13 expression pattern by this method in *S. coelicolor* will also be important in deciding whether mRNA levels detected using RT-PCR correspond to actual protein levels. This will be useful in addressing whether mRNA stability is a mode of regulation for expression of the putative helicase gene as has been noted for cold shock proteins in *E. coli* (Goldenberg *et al.*, 1996). Furthermore, purifying the putative helicase enzyme will enable biochemical analysis and characterization of the SCH5.13 gene product. It has been pointed out that the presence of the

conserved amino acid motifs characteristic of RNA helicases does not confirm helicase enzyme activity and so utilizing purified SCH5.13 protein for biochemical assays will verify whether the enzyme can act as an RNA helicase *in vitro*. In addition, it can be determined whether this protein requires ATP for energy or whether it has a preference for another NTP.

Detecting the SCH5.13 transcript in total RNA preparations harvested from *S. coelicolor* proved to be a challenging endeavor. The only success in determining the expression pattern was in the use of RT-PCR, which amplifies the transcript to a detectable level using PCR. Using this method, a dependence on *S. coelicolor* growth phase was established with the transcription peaking at 36 hours post-inoculation. This harvest time corresponds to the onset of spore formation in *S. coelicolor*, which implies a role for this putative enzyme in this process; however, the mRNA could be detected at all time points examined and interestingly, a peak in transcript levels was also noted in the 15 hour, vegetative mycelium sample. It should be pointed out that transcript levels may not correspond to the level of SCH5.13 protein present, nor does it give any indication of whether the enzyme would be active at this time. Often genes encoding proteins required for transient processes such as nutrient stress or temperature shock are transcribed throughout the life span of the organism, but differential mRNA stability offers a translational regulatory element on top of the temporal transcriptional control. Examination of the protein expression pattern utilizing antibodies raised against purified SCH5.13 gene product should help determine whether this phenomenon exists with SCH5.13 expression.

Confirmation of the involvement of SCH5.13 in the development of *S. coelicolor* lies in the identification of the target(s) of its activity. The close proximity of the helicase gene to the divergently transcribed *bldG* offers this putative anti-anti sigma factor gene as a potential target. The transcription start site of SCH5.13 was localized to a small region that overlaps the −10 sequence of the *bldG* P1 promoter, which suggests a possible coordinate control mechanism for transcription regulation of these two genes. In *E. coli*, competition for the RNA polymerase enzyme can act as a method for controlling transcription of one or both of the divergently transcribed genes (Farewell *et al.*, 1998). In addition, it is possible that the relatively long (>100 bp) 5' untranslated region (UTR) that results if the ATG translation start codon discussed above is correct is necessary for translational regulation of SCH5.13 expression as has been found in the cold shock proteins in *E. coli* (Jiang *et ai.*, 1996). Although initial examination of this region has revealed no consensus binding sequence or solid evidence supporting relevant secondary structure, its presence does merit further investigation.

Not only is there the potential that *bldG* and the putative RNA helicase gene SCH5.13 are transcribed utilizing a divergent coordinate control mechanism, but the *bldG* locus itself presents a reasonable need for an RNA unwinding enzyme. Investigations by Bignell *et al.* (2000) have identified two potential stem-loop structures in the intergenic region between *bldG* and the downstream co-transcribed *orf3* gene. It is possible that this secondary structure in the *bldG* operon mRNA serves as a target of helicase activity, however, what

role unwinding would play in development of *S. coelicolor* is currently unknown. However, two possible scenarios present themselves.

The first plausible scenario is that the RNA helicase would unwind the stem-loop secondary structure in order to promote degradation of the *bldG* monocistronic transcript and thus prevent translation of the anti- anti sigma factor (Fig. 4.1). This would result in binding of the sigma factor by the anti sigma factor (ORF3) and presumably prevent the transcription of those genes necessary to initiate aerial hyphae formation and sporulation. In this situation, expressing the helicase earlier in the life cycle, or at a higher copy number, would result in premature degradation of the *bldG* transcript and thus should lead to a bald phenotype. In fact, when the helicase gene was present in pAU228 which integrates into the *S. coelicolor* genome, there was a noticeable delay in the development of aerial hyphae. The colonies eventually did sporulate which may be explained by the abundance of the *bldG* transcript when compared to the SCH5.13 RNA. Eventually the anti-anti sigma factor protein would reach a level where it could overcome the activity of the RNA helicase / RNAse complex and allow differentiation to continue as normal.

Still assuming that this scenario where the function of the helicase is to promote degradation of the *bldG* transcript is correct, disrupting its enzymatic activity by creating a null mutant would result in early translation of the anti-anti sigma factor. The premature presence of this protein would allow early release of the sigma factor and thus result in untimely expression of aerial hyphae or sporulation genes, which could be fatal to the organism. Again, this situation is

Figure 4.1: Schematic drawing of the putative RNA helicase as a negative regulator of *bldG* expression. (A) Two *bldG* containing transcripts with their respective promoters represented by P1 (monocistronic *bldG* transcript) and P2 (polycistronic *bldG-orf3* transcript). Location and structure of stem-loop structures is approximate. (B) The putative RNA helicase associates with an RNAse enzyme to unwind the secondary structure at the 3' end of the *bldG* monocistronic transcript, thereby promoting degradation of the mRNA transcript. *bldG* and *orf3* are represented by blue and red arrows respectively. The RNAse enzyme is shown as a yellow oval and the RNA helicase enzyme is represented by the multi-colour circular arrows marked H.

A

Monocistronic

Polycistronic

B

Helicase unwinds secondary
structure so RNAse can
digest RNA

RNAse

H

bldG transcript degraded - no
translation of anti - anti - sigma
factor
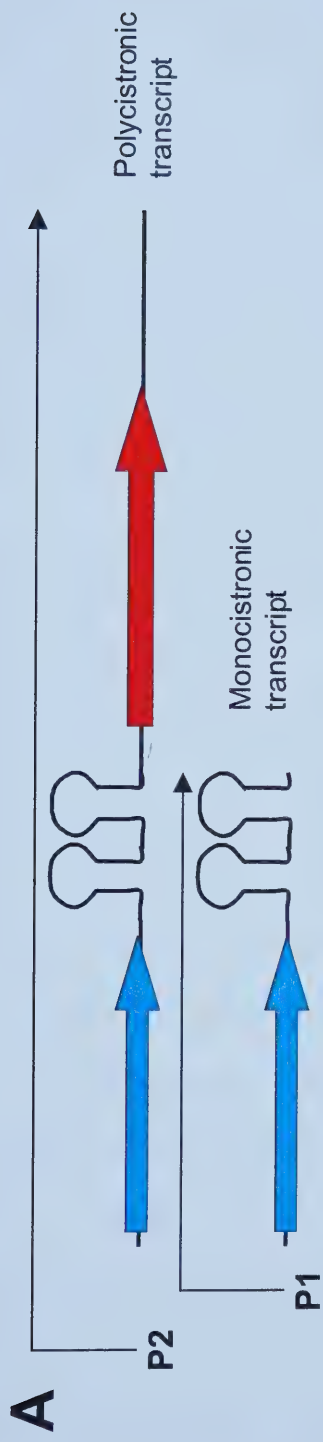
supported by the inability to create a null mutant in a wild-type strain of *S. coelicolor*. Furthermore it could help to explain the ability to disrupt the helicase gene in a *bldG* mutant strain. A study of the *nrsA* gene (formerly *orf1590*) in *S. griseus*, (McCue *et al.*, 1996) revealed a similar phenomenon in which a deletion of the gene was only possible in a strain that harboured a second-site bald mutation. It was concluded that the product of this gene was a negative regulator of sporulation and that deleting the gene resulted in premature septation and death. Similarly, in *Bacillus subtilis* disruption of the anti sigma factor, *spoIIAB*, was only possible in a strain harbouring a mutation in the sigma factor, σ^F, which made the prematurely released product less toxic to the cells (Coppolecchia *et al.*, 1991). One method of testing this theory would be to measure the abundance of *bldG* mRNA in the 'double helicase' strain of *S. coelicolor* relative to the wild type. The number of *bldG* transcripts would be expected to be vastly reduced in this strain if the helicase function was to degrade the monocistronic transcript.

How the anti-sigma factor transcript would be translated and not degraded by the same RNAse that would be digesting *bldG* transcript is unclear, but perhaps the answer will be clarified once the necessity of expressing two *bldG*-containing transcripts is determined (Bignell *et al.*, 2000). It is possible that the secondary structure found at the end of the polycistronic transcript is not recognized by the putative RNA helicase – RNAse complex therefore the monocistronic transcript is the only target of the helicase and thus RNAse activities. This would still result in a 1:1 stoichiometry of BldG:ORF3 proteins

early during colony development, however perhaps the BldG protein is inactive until it is necessary for the sigma factor to be released to direct transcription of its target gene(s). Also unexplained by this theory is the abundance of helicase transcripts present later, rather than earlier, in the *S. coelicolor* life cycle. It would be expected that if the function of the helicase were to aid in the degradation of the *bldG* transcript early during colony development then it should be expressed early in the life cycle and expression should decline as the anti-anti sigma factor is required for sporulation. While the SCH5.13 ORF was transcribed at high levels during vegetative growth (the 15 hour time point sample), its expression also peaked much later at approximately the same time as does *bldG* transcription. These results have necessitated an alternative explanation for the function of this putative RNA helicase.

Instead of being a negative regulator of *bldG* expression, it is possible that the putative RNA helicase SCH5.13 is a positive regulator of *orf3* expression. The secondary structure formed by the stem-loops in the *bldG-orf3* intergenic region could be serving as a rho-independent termination signal. If the anti sigma factor cannot be transcribed, then the protein cannot be translated and so perhaps the putative RNA helicase, SCH5.13, is required to unwind this secondary structure to promote *orf3* transcription and subsequent translation. Alternatively, the entire polycistronic transcript could be transcribed efficiently, however, secondary structure in the *bldG-orf3* intergenic region could cause the ribosome to pause during translation, thus disallowing anti sigma factor protein expression. The putative RNA helicase could be necessary to unwind this and

so promote translation of *orf3* transcripts (Fig. 4.2). In this same vein, the

secondary mRNA structure could be associated with a number of RNA

associated proteins thus forming a ribonucleoprotein (RNP) complex as has been

recently described for the DExH RNA helicase, NPH-II in Vaccinia virus

(Jankowsky *et al.*, 2001). It is possible that the putative RNA helicase encoded

by SCH5.13 serves to dislodge the proteins from this RNP complex, thus making

it a putative "RNPase" (a term coined by Jankowsky *et al.*, 2001), much like

NPH-II, that could promote translation of the *orf3* gene. If the putative RNA

helicase encoded by SCH5.13 did act in this manner, its activity would be

expected to be highest during vegetative growth and then decline by ~24 hours

when aerial hyphae appear. At this stage the anti sigma factor is presumably

inactivated by the BldG anti-anti sigma factor binding, which would release a

sporulation-specific σ factor to transcribe genes required for morphological

development. However, RT-PCR experiments have shown repeatedly that

transcription of the RNA helicase occurs throughout the life cycle yet increases

later on, which appears to contradict this theory. On the other hand, in keeping

with the need to have an RNA helicase expressed during early colony

development if this theory were to be correct, a surge of helicase transcription

appears to occur in the 15 hour vegetative mycelium samples and this is followed

by a decrease soon after. Perhaps the initial surge of helicase expression is

enough to allow translation of the anti sigma factor in the early stages of colony

development. This surge, together with the possibility that the BldG protein's

association with the anti sigma factor is regulated post-translationally (Bignell *et*

Figure 4.2: Schematic drawing of the putative RNA helicase as a positive regulator of *orf3* translation. (A) The putative helicase enzyme associates with a ribosome during *bldG-orf3* translation. The stem-loop structures in the *bldG-orf3* intergenic region cause the ribosome to pause before translation of the anti-sigma factor. Helicase enzyme activity unwinds the stem-loop structures, thus allowing efficient translation of *orf3*. *bldG* and *orf3* are represented by blue and red arrows respectively. The ribosome is shown in pink and the putative RNA helicase is shown as multi-coloured circular arrows marked H.

A

B

Rb

H

Rb

H

Anti σ factor

BldG
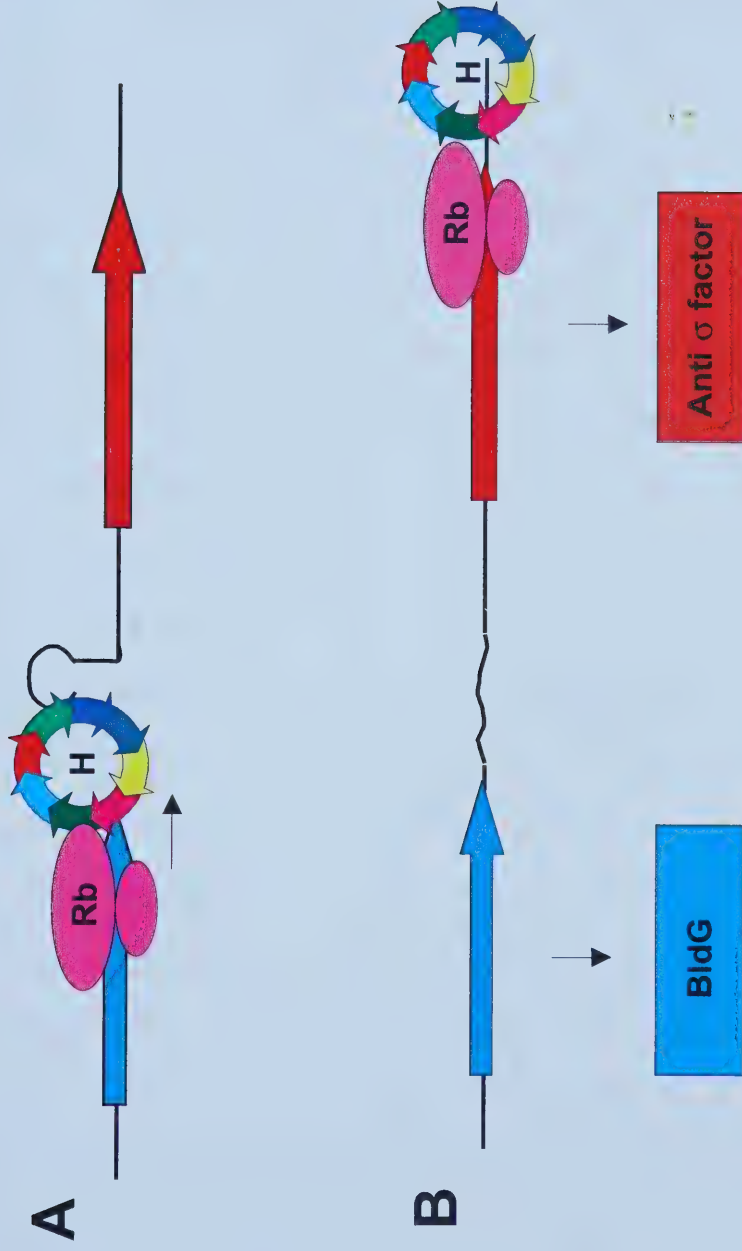
al., 2000), would render the sigma factor responsible for transcribing the unknown genes required for development, inactive. Under these circumstances, if the putative helicase were present in abundance, as it is with an extra copy of the SCH5.13 gene integrated into the genome, there would be a resulting increase in the amount of anti sigma factor translated. As a result of extra anti sigma factor present, the sigma factor would not be released until such time as BldG levels could rise to a sufficient level to overcome the repression. Again, the developmental delay in *S. coelicolor* harbouring pAU228 could be a result of this phenomenon. If the anti sigma factor RNA was unable to be translated, as would be the case in this scenario in a putative helicase null mutant, the sigma factor would be free at all times to initiate transcription of the genes it controls. This situation could result, just as in the case of an *nrsA* null mutant, in premature septation and subsequent death of the organism unless there was a second site bald mutation. In this case this was achieved by using a *bldG* mutant as a host for SCH5.13 gene disruption. With no anti-anti sigma factor, there would never be a release of the sigma factor and so, at first thought, mutating the helicase would make no difference to the organism. However, a mutation in the helicase should still prove fatal if this scenario is correct because without it the anti sigma factor could not be translated and would never have been associated with the sigma factor. Therefore, sporulation specific genes would be expressed during vegetative growth, presumably leading to colony death.

It is clear from the evidence gathered so far that neither of the above explanations can serve as the sole answer to the question posed by the

presence and location of the putative RNA helicase gene and so an interesting

possibility presents itself. Could this enzyme regulate both *bldG* degradation and

orf3 translation? RNA helicases have been found that associate with ribosomes

in order to promote more efficient translation of mRNA secondary structure when

the situation warrants it. An example of one such enzyme being the *E. coli* cold

shock RNA helicase, CsdA, which is believed to associate with the ribosome

during cold shock where decreased temperatures would result in increased

stability of mRNA secondary structure (Jones *et al.*, 1996). There have also

been RNA helicases described in association with RNAses such as RhlB found

in complex with RNAseE and PNPase in the *E. coli* degradeosome (Py *et al.*,

1996; Sommerville, 1999). Not only are RNA helicases known to associate with

other proteins, they can also be translated as part of a larger, multi-domain

protein with RNA helicase activity being restricted to just one domain. An

example is the developmentally significant RNA helicase, Caf, found in

Arabidopsis thaliana (Jacobsen *et al.*, 1999). Caf is actually just the N-terminal

portion of a larger protein that also contains an RNAseIII-like domain in the C-

terminal region. That both Caf and the putative *S. coelicolor* RNA helicase

SCH5.13 contain the DECH version of the DEAD-box motif is interesting, as this

motif is unusual, having only been found otherwise in the Hepatitis C Virus (HCV)

RNA helicase (Kim *et al.*, 1997). Also of interest is *absB,* a pleiotropic regulator

of antibiotic biosynthesis in *S. coelicolor*. The product of *absB* has been

identified as an RNAseIII enzyme, indicating that RNAses do have a role in

streptomycete differentiation (Price *et al.*, 1999).

Unfortunately, no one RNA helicase enzyme has been described in the literature that is found both as part of an RNAse-containing complex and associated with aiding ribosomal translation of mRNA. Is it possible that modifications of protein structure could occur which allow the enzyme to act in different capacities depending on the either environmental conditions or phase of growth? In an organism that is as developmentally complex as *Streptomyces*, is it not reasonable to hypothesize a multi-functional role for this enzyme? In fact, in the absence of a defined target, it is possible that this putative RNA helicase could be a key regulator of a number of genes involved in morphological or physiological differentiation. The RNA helicase Vasa, found in *Drosophila*, has been shown to positively regulate translation of *nanos* mRNA which is important for abdominal development, possibly by unwinding secondary structures in the mRNA that would otherwise impede translation (Gavis *et al.*, 1996). Similarly, in *E. coli* many genes involved in the cold shock response possess a 5' untranslated region that contains a conserved recognition sequence called the "cold box" that is recognized by various proteins and has been shown to regulate transcription of genes required for this response (Jiang *et al.*, 1996). Perhaps a common secondary structure or a conserved recognition sequence is present in genes required for differentiation and this helicase is responsible for regulation of their translation. In this vein, construction of a recombinant plasmid that can function to identify potential helicase targets via microarray technology is underway. This technology uses a thiostrepton-inducible promoter that is used to drive expression of the SCH5.13 gene which will result in overexpression of the

protein. Presumably the SCH5.13-encoded putative helicase protein will aid in translation of one or more target genes, the resulting proteins of which could potentially activate transcription of their target genes, thus revealing indirect targets of helicase activity. In identifying genes indirectly activated by SCH5.13 activity, we can then attempt to elucidate the direct target(s). One method could be to use DNA sequences from the gene(s) found using microarray experiments, to probe *S. coelicolor* cell free extracts in gel retardation experiments. Proteins that impede migration of the DNA sequences through a polyacrylamide gel could then be purified and subsequently analyzed as was done to identify BldD as a regulator of *sigH* expression (Kelemen *et al.*, 2001). Alternatively, a proteomic approach could be utilized with the same recombinant plasmid in which proteins present under thiostrepton promoter – inducing (increased SCH5.13 activity) verses non-inducing (decreased SCH5.13 activity) conditions can be compared using 2-dimensional gel electrophoresis. Many of the questions raised throughout this thesis project can potentially be answered once the genes that depend, either directly or indirectly, on SCH5.13 expression are identified. However, as problems with overexpressing this putative gene in *S. coelicolor* have occurred during the course of this project, it is a possibility that the effects of using this plasmid will be too deleterious to gain useful information. However, this result in itself would be beneficial in solidifying the role of this putative RNA helicase in *S. coelicolor* development.

One point that also bears consideration is the possibility that the SCH5.13 gene product plays both a developmental role and a vegetative one. The

presence of a high amount of putative RNA helicase transcript consistently in the

15 hour sample is indicative of a vegetative growth requirement for this protein.

The GTP binding protein, Obg, in *S. coelicolor* has been implicated in

developmentally regulated sensing of the GTP pool throughout the life cycle,

however, attempts to disrupt the gene have proved unsuccessful and has thus

led researchers to propose that Obg also has a purpose during vegetative growth

(Okamoto and Ochi, 1998). Interestingly, a few-fold increase in *obg* expression

impaired development of aerial mycelium without affecting vegetative growth.

Although it may prove to be possible to disrupt SCH5.13 using a technique other

than protoplast transformation, it is not unrealistic to expect that it may never

occur. Several RNA helicase genes, including *rhlB* from *E. coli* and *mle* in

Drosophila, have proved to be essential for viability despite having very different

functions within their host organisms. It would strengthen the argument that

disruption is lethal in wild-type *S. coelicolor* if a crossover could be obtained in

the strain harbouring a second copy of SCH5.13. Although the attempts in this

thesis have been unsuccessful because of the instability of the strain, it could be

possible if the second copy of SCH5.13 were functionally repressed by placing it

under the control of the *gyl* promoter, as has been done during analysis of the

heat shock regulon in *S. coelicolor* (Bucca *et al.*, 2000). The *gyl* promoter is

capable of being repressed by glucose and induced by glycerol, and so by

growing the strain with two SCH5.13 genes on glucose, it may be possible to

cultivate the strain and create protoplasts which can then be transformed with the

disruption vector, pAU226.

Finally, because *Streptomyces* undergo transformations into vastly different morphological structures that require proper temporal and spatial expression of a complex network of signals, it is attractive to suggest that the putative RNA helicase SCH5.13 could play a role in development similar to that of *Drosophila*'s Vasa. Localization to specific structures has been confirmed for numerous developmentally important genes in *Streptomyces* including *bldA* (Kataoka *et al.*, 1999), *redD* (Sun *et al.*, 1999), and the late sporulation-specific sigma factor, *sigF* (Sun *et al.*, 1999; Kelemen *et al.*, 2001). The SCH5.13-encoded enzyme may have targets in specific structures at different times during colony development. For example, since 18 hours corresponds to a transition phase between substrate mycelial growth and aerial hyphae development, perhaps the decrease in SCH5.13 then allows BldG to be active, meaning that the sigma factor is active, allowing aerial hyphae to form. The second peak of helicase activity could presumably occur only in aerial hyphae where BldG/ORF3 might not be expressed and so this second peak is aimed at different genes. Utilizing an SCH5.13 – GFP (green fluorescent protein) fusion and visualizing where the putative helicase protein is expressed throughout the life cycle will be a useful tool to determining if this is a real possibility for how this potentially key enzyme functions in *S. coelicolor* development.

Although much of the speculation as to the role that the SCH5.13-encoded putative RNA helicase plays in *S. coelicolor* development has centered around the idea that *bldG*, with its close proximity and overlapping promoters, is a target of the enzymatic activity, the possibility exists that it is not. There has been little

to no success with finding definitive *in vivo* targets of RNA helicase activity reported in the literature due to the fact that these enzymes usually act at the level of translation, thereby necessitating the use of a proteomics approach as described above.

In conclusion, the outcome of this research has been successful in that the putative RNA helicase encoded by the SCH5.13 ORF has been identified as having potential involvement in differentiation in *S. coelicolor*. Although the work completed in this study has not identified a defined target of the enzyme's activity, it has recognized a new element in development previously uncharacterized in streptomycetes. The potential presence of RNA helicases in the developmental cascade of *S. coelicolor* is an exciting possibility that should help further unravel the complex networks necessary for morphological and physiological differentiation in these complex organisms.

Chapter 5:

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